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Variation in Mitochondrial DNA (mtDNA) in Western Canadian Populations of Lake Trout, *Salvelinus namaycush*, and Rainbow Trout, *Oncorhynchus mykiss*.

By

Renato Vitic ©

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

DEPARTMENT OF ZOOLOGY

Edmonton, Alberta
Spring 1995

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R2G 4A3

Date: Jan 30/95

" And the truth is that as a man's real power grows and his knowledge widens, ever the way he can follow grows narrower: until at last he chooses nothing, but does only and wholly what he *must* do ..."

—*A Wizard of Earthsea*

Ursula K. Le Guin

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Variation in Mitochondrial DNA (mtDNA) in Western Canadian Populations of Lake Trout, *Salvelinus namaycush*, and Rainbow Trout, *Oncorhynchus mykiss*" submitted by Renato Vitic in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

The sequence variation and diversity of mitochondrial DNA (mtDNA) in members of the family Salmonidae were examined at the species, population, and individual levels, to examine the utility of mtDNA for stock identification of members of the Salmonidae. Sequence comparisons were made of two regions of the mtDNA from members of the genus *Salvelinus*; *S. namaycush* (Walbaum), *S. confluentus* (Suckley), and *S. fontinalis* (Mitchill). Comparisons of sequence data from the non-coding control region, and a region spanning two protein coding genes, the ATPase subunit 6 gene (ATPase 6) and the cytochrome oxidase subunit III gene (COIII), surprisingly revealed similar levels of genetic divergence between the non-coding region and the two coding genes. Phylogenetic analysis of the same sequences indicated a closer evolutionary relationship between *S. namaycush* and *S. confluentus* than of either to *S. fontinalis*. The mtDNA diversity in populations of lake trout, *Salvelinus namaycush*, in west central Canada was examined using both restriction fragment length polymorphism (RFLP) analysis and DNA sequencing of the ATPase-6/COIII region. Genetic analysis of *Salvelinus namaycush* from 10 populations identified a total of 17 haplotypes. Twelve haplotypes were unique to one of the ten populations but, five haplotypes were common, occurring in more than one population. The occurrence of five mtDNA lineages in more than one population suggests a recent origin of these populations with little genetic divergence since their geographic separation. Recent human transfers between populations may account for the co-occurrence of types between populations, but analysis of mtDNA failed to unambiguously assign individual fish to a particular population. Restriction fragment analysis of the control region, and the ATPase-6/COIII region of 40 individual rainbow trout, *Oncorhynchus mykiss* (Walbaum), from 8 populations, consisting of both hatchery reared and local rainbow trout, revealed a total of three haplotypes. None of the haplotypes observed were unique to any one of the populations examined and stock differentiation of individual rainbow trout was not possible.

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Introduction

The Problem

Few organisms, with the possible exception of agricultural crops and domesticated livestock, have undergone such heedless and widespread manipulation as freshwater fish stocks (Allendorf, 1987). Manipulation of wild fish stocks includes the introduction of new stocks of fish, supplemental stocking of hatchery reared fish to exploited wild stocks, and the removal of undesirable fish populations that may inhibit the establishment of more desirable fish species. These manipulations have in the past been initiated for the express objective of providing the "optimum yield" in recreational and commercial fishing (Allendorf, 1987).

The earliest record for the introduction of an exotic freshwater fish species to North America dates back to the late 1600's when goldfish, *Carassius auratus* were introduced to New York State (Dekay, 1842, Crossman, 1991). Approximately 143 fish species, hybrids, and strains are recognized to have been introduced or transferred within Canada alone (Crossman, 1991). In particular, salmonid species have been subjected to extensive introductions and transfers both within and between continents. The only successful establishment of an exotic salmonid to North America was that of the European brown trout, *Salmo trutta* (Krugger and May, 1991). Extensive introductions and transfers of salmonid species both within and outside of their natural ranges in North America have occurred for all members of the genera *Oncorhynchus*, *Salvelinus*, and *Salmo* (Nelson and Paetz, 1992; Mayhood, 1992; Crossman, 1991; Krugger and May, 1991). This was due primarily to the refinement of methods for the collection and hatching of large numbers of trout eggs during the late 19th century. This resulted in what Behnke (1992) refers to as the "Johnny Appleseed era" in fisheries management. The extensive numbers of past introductions and transfers of fish species both within and outside of their ranges in Canada have resulted in a biological jigsaw puzzle of naturally occurring and introduced stocks of fish.

Previous attempts to enhance the "optimum yield" have often been initiated without first establishing methods to evaluate the impact of these activities on introduced and resident fish species, non-commercial fish species, amphibians, and invertebrates (Allendorf, 1991; Philip, 1991). The impact on the genetics of resident fish populations and introduced fish is another area that has largely been ignored (Allendorf, 1987). Clearly the effects of fish introductions, over-harvesting, and mixing of different fish stocks, on the genetics of fish species and populations are poorly understood.

The preservation of gene pools is as an important factor for the maintenance of genetic diversity and adaptive potential of fish stocks (Allendorf *et al.*, 1987). Human activities have greatly influenced the distribution and abundance of fish species. Factors such as poor adaptability of genetically dissimilar stocks, inbreeding, introgression, and loss of genetic diversity may contribute to the deterioration and the possible extinction of unique stocks of fish (Mayhood, 1992; Crossman, 1991; Waples, 1991). The influence of these factors on the gene pools of affected fish species and populations is unclear and has created a need for the genetic characterization and monitoring of fish species and populations (Allendorf, 1991).

Gyllensten and Wilson (1987) summarized the general objectives of genetic analysis of fish populations:

> 1) The detailed description of the genetic structure of natural and hatchery propagated populations.
> 2) The development of genetic markers that enable single fish to be assigned unambiguously to a specific hatchery strain or natural population.
> 3) Provide systems for the monitoring of quantitative and qualitative changes in the genetic resources resulting from the various management activities (for example, sensitive methods for estimating the loss of genetic variation in both hatchery and natural populations).
> 4) The patenting of unique strains of hatchery propagated, and genetically engineered fish species.

Methods to characterize and monitor genetically distinct populations may then be applied to assess the impact of activities such as over harvesting, introduction, transfer and mixing of fish stocks upon the gene pools of specific fish populations and species (Billington and Hebert, 1991; Gyllensten and Wilson, 1987).

Lake trout, *Salvelinus namaycush* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), are two popular sport fish broadly distributed across Canada (see Figure 1 and 2). These species have undergone extensive supplemental stocking and over-exploitation across their entire range (Mayhood, 1992; Crossman, 1991), including the prairie provinces (Nelson and Paetz, 1992; Roberts, 1975; Dietz, 1967). Historical mixings of wild stocks and the introductions of hatchery strains have resulted in confusion over the origins of lake trout and rainbow trout populations in Alberta (Mayhood, 1992; Donald, 1987). Therefore the need to characterize the genetic variation of existing populations of these two species is vital for understanding their current distributions and the impacts of past manipulation.

The purpose of this thesis was to first examine differences in the genetic structure of two regions of mtDNA, the non-coding displacement loop or control region, and a region spanning two protein coding genes, the ATPase subunit 6 (ATPase 6) and the cytochrome oxidase subunit 3 (COIII), of three members of the genus *Salvelinus*: the lake trout, *S. namaycush* (Walbaum); the bull trout, *S. confluentus* (Suckley); and the brook trout, *S. fontinalis* (Mitchill). Second goal was to compare the levels of mtDNA variation between populations of two Salmonid species, the lake trout, *Salvelinus namaycush*, and the rainbow trout, *Oncorhynchus mykiss* (Walbaum), in west-central Canada. Populations of both native and introduced fish were examined to describe the genetic structure of both natural and artificially established populations of *S. namaycush* and *O. mykiss*. Eight populations of lake trout, distributed across Alberta, one population in Saskatchewan and a single population in Manitoba were examined. The individuals

Figure 1. The distribution of lake trout, *Salvelinus namaycush*, in Canada with particular emphasis on west-central Canada. The shaded regions correspond to the distribution of lake trout; modified from Scott and Crossman (1973), and Nelson and Paetz (1992).

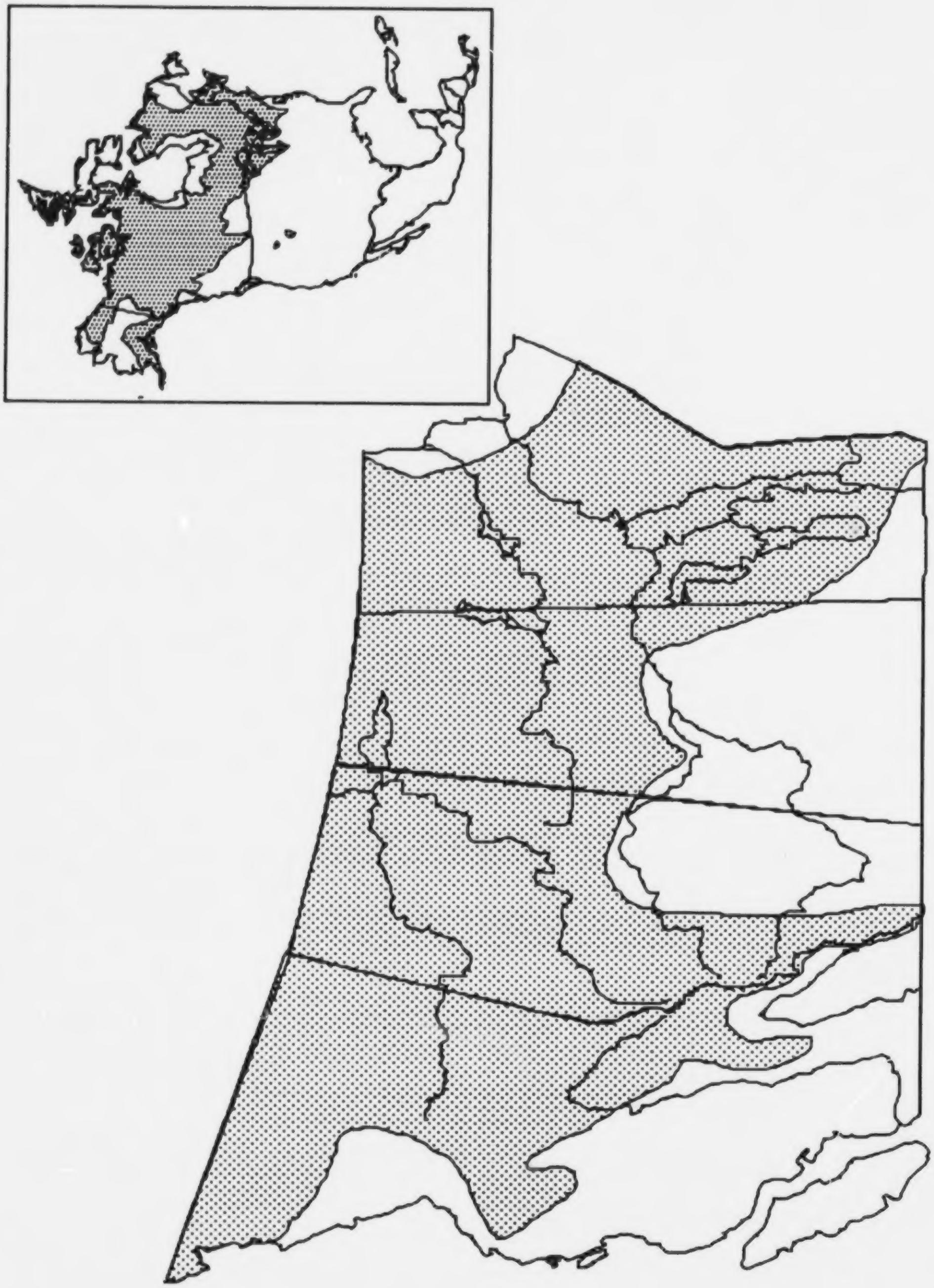
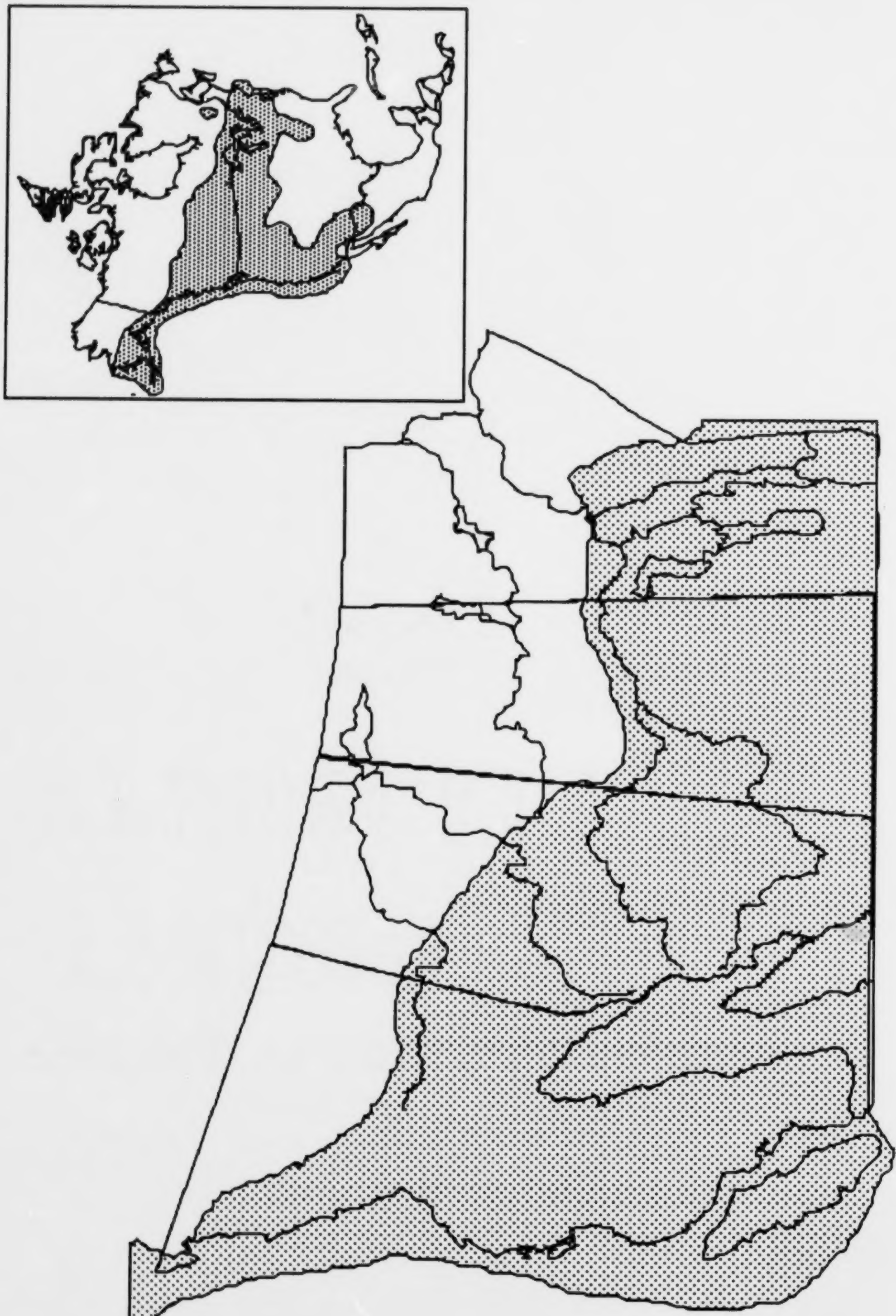


Figure 2. The distribution of rainbow trout, *Oncorhynchus mykiss*, in Canada with particular emphasis on west-central Canada. The shaded regions correspond to the distribution of rainbow trout; modified from Scott and Crossman (1973), and Nelson and Paetz (1992).



from the two populations in Saskatchewan and Manitoba, and a single population from Alberta were historically used as source lakes for the supplemental stocking of lake trout to Cold Lake, Alberta. These populations were compared to the resident population within Cold Lake to distinguish between native and introduced fish. Individuals from the Wampus Creek population of Athabasca rainbow trout were compared to rainbow trout from local populations distributed across Alberta, to several individuals from two local hatchery strains, and to individuals from a genetically similar hatchery strain from British Columbia.

Molecular Analysis of Fish Populations

The distributions of modern day fish populations have resulted from the historical colonization of their extant ranges following the retreat of the Pleistocene ice (reviewed by McPhail and Lindsey, 1970; Mayhood, 1992). However the "natural" patterns of variation in species such as lake trout and rainbow trout have been obscured by past and current human manipulation. Several attempts have been made to reconstruct the past distributions of fish species. Paleolimnological records have been used to establish the presence or absence of plankitivorous fishes and changes in fish and their supporting communities of organisms (Miskimmin and Schindler, 1994; Lamontagne, 1993). Morphological and meristic studies of conspecific fish populations form the basis for the correlation of past and present day distributions (Behnke, 1979; Sterling, 1989). Recent limnological surveys have been compared with archived information to reconstruct stocking histories and to assess long term impacts of fish introductions (Mayhood, 1992).

Modern molecular methods provide another approach to the reconstruction of the past. By examining the DNA of individuals from extant fish populations another historical record is exposed — a record of mutational events and gene flow between populations that provides a historic view of populations and strains, subspecies and higher taxa by the composition of their respective genomes (see Utter, 1991 for a historical review). Several methods for the analysis of variation in the DNA of individuals are available. The discussion here however, will be limited to those methods used in this thesis.

The advent of the polymerase chain reaction or PCR has greatly facilitated the isolation, replication, and detection of variation in specific regions of DNA (for a comprehensive review of the technology see Innis *et al.*, 1990). The PCR involves the enzymatic amplification of minute quantities of specific fragments of DNA through the use of a thermally stable polymerase (an enzyme that replicates DNA), specific oligonucleotide primers (short fragments of single stranded DNA used to initiate synthesis by the polymerase), and a series of temperature cycles (denaturation, annealing and synthesis stages). Through this method large quantities of specific regions of DNA may be produced in a relatively short period of time.

Following the amplification of homologous regions of DNA for two or more specimens, the number of genetic or nucleotide differences within these homologous regions can be detected by both restriction fragment length polymorphisms (RFLP), and direct DNA sequencing. Restriction enzymes are enzymes that recognize specific sequences of DNA, and cut double stranded DNA wherever such sequences occur. The number of nucleotide base pairs in a recognition sequence may vary from four to six or more base pairs, depending upon the enzyme used. By RFLP analysis homologous regions of DNA are digested with an individual restriction enzyme and the resulting fragments are separated according to size by gel electrophoresis, and stained with a fluorescent compound to visualize the restriction fragment patterns. Restriction fragments of similar size are assumed to be identical, and size differences between restriction patterns indicate nucleotide changes at the restriction sequence site of that particular enzyme. This method of detecting genetic differences is favored for its relative ease and low cost, but the analysis of differences is restricted to the recognition sequences of the different restriction enzymes used. The resolving power of restriction enzymes is limited because any differences that occur outside of the restriction sites of the various enzyme used remain undetected.

Direct DNA sequencing allows examination of each individual nucleotide in a strand of DNA and offers the greatest resolving power for the detection of variation in DNA. In the past, studies using DNA sequencing were constrained by the difficulty and cost associated with traditional cloning technology making large scale surveys of populations difficult. The PCR has greatly simplified this process and, as a result, sequencing has become more widely adopted for the molecular characterization of fish species and populations (Meyer *et al.*, 1990; Meyer, 1994). However it remains relatively costly for the genetic survey of large numbers of individuals.

A combination of the two methods described above provides a powerful strategy for the genetic characterization of large numbers of individuals from diverse populations. The studies in this thesis take advantage of the utility of RFLP analysis for the genetic survey of large number of individuals, and the resolving power DNA sequencing for clarifying phylogenetic relationships to examine the genetic diversity of populations of lake trout, *Salvelinus namaycush*, and rainbow trout, *Oncorhynchus mykiss*.

Mitochondrial DNA

One type of DNA that has become widely adopted for the examination of "genetic distances" between fish populations, subspecies and species is mitochondrial DNA. Several characteristics of mtDNA make it the "molecule of choice" for the examination of genetic differences between closely related taxa. Mitochondrial DNA (see Figure 3) occurs in the mitochondria of cells and exists as a single set (haploid), circular molecule that is inherited maternally (Ferris and Berg, 1987; Beckenbach, 1991). Furthermore, mtDNA does not undergo recombination. In mammals it evolves 5 to 10 times more rapidly than nuclear DNA (Brown *et al.*, 1979). Thus, matriarchal lineages based on mtDNA may diverge faster than nuclear DNA, and are uncomplicated by crossing over and assortment

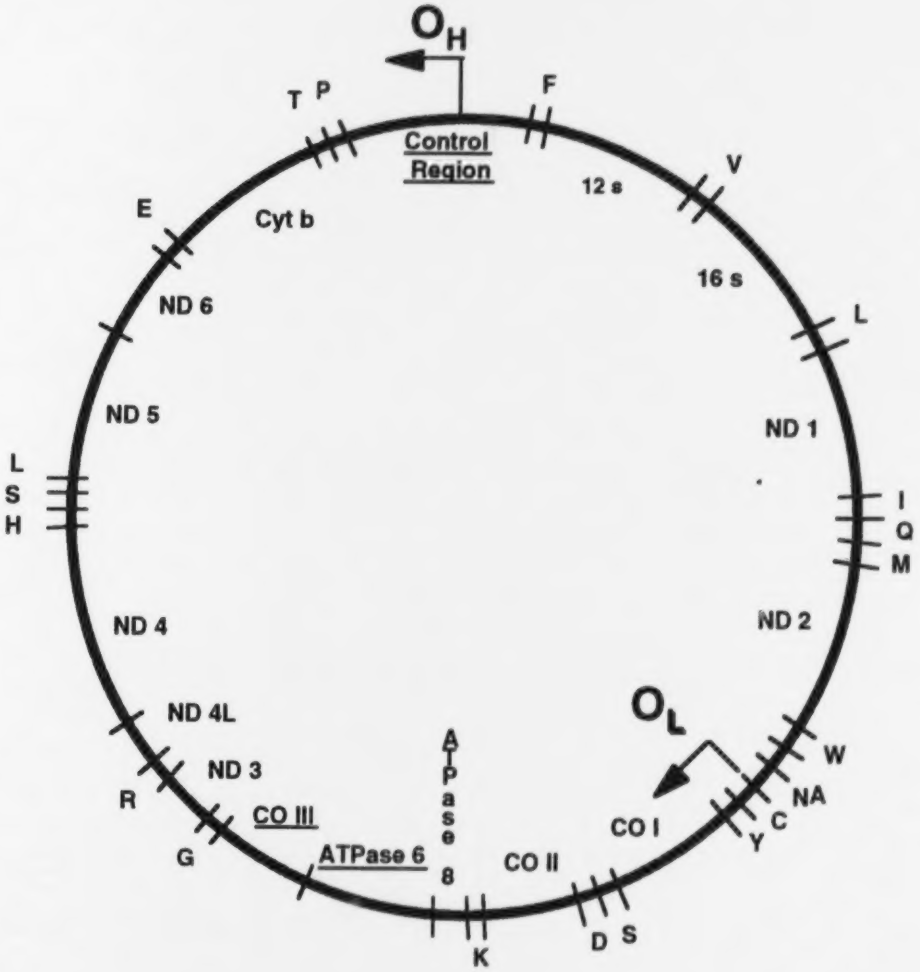

O_H
T
P
F
Control Region
12 s
V
E
Cyt b
16 s
L
ND 6
ND 5
ND 1
L
S
H
I
Q
M
ND 4
ND 2
O_L
ND 4L
W
A T P a s e 8
ND 3
NA
R
CO III
C
CO I
Y
G
ATPase 6
CO II
D
S
K

Figure 3. The vertebrate mitochondrial DNA map adapted from Thomas and Beckenbach (1989), and Allendorf (1991). Protein, rRNA coding genes and the non-coding control region are labeled on the inside of the map. The tRNA genes are depicted by their single letter amino acid codes adjacent to their position on the outside of the map. The position of the origins of heavy (O_H) and light (O_L) strand replication are denoted by arrows.

events that occur in nuclear DNA (Lansman *et al.*, 1981). Restriction digestion or RFLP analysis of total mtDNA has been useful for estimating rates of sequence divergence among congeneric fish taxa (Meyer 1994), genetic relationships among fish species (Gyllensten and Wilson, 1987; Avise and Saunders 1984), salmonid subspecies (Gyllensten *et al.*, 1985), and fish populations (Grewe and Hebert, 1988).

The development of the PCR has facilitated genetic studies of rapidly evolving regions of mtDNA, rather than of the entire mtDNA molecule. Different regions of mtDNA evolve at different rates and genetic analysis of evolutionary "hot spots" could facilitate the rapid analysis of large numbers of individuals with greater resolution between closely related groups (Beckenbach, 1991). Comparisons of the rates of mutational change in different regions of mammalian mtDNA indicate that the highest rates are localized to the untranscribed D loop region (Brown *et al.*, 1981), intermediary rates occur in protein coding genes (Thomas and Beckenbach, 1989), and the lowest rates of mutational change were found in genes that code for RNA's (Ferris and Berg, 1987; Cann, *et al.* 1984).

Given the patterns of nucleotide substitution observed in mammals, the control region, as the only non-coding region of the mtDNA, should give the highest degree of resolution in detecting and characterizing genetic differences between species. One disadvantage in using a non-coding region such as the control region for phylogenetic analysis is that the alignment and therefore positional homology across different taxa is not constrained by a protein product. Changes in the nucleotide sequence may overlap with deletions and insertions thereby resulting in ambiguity over the alignment of homologous regions between taxa, and subsequent errors in the generation of the phylogenies of these taxa.

Protein coding regions offer an alternative to non-coding regions. Amino acids are specifically coded for by adjacent non-overlapping triplets of nucleotides called codons, and most amino acids are coded for by more than one codon. Codons that code for the same amino acid are called synonymous codons, and nucleotide changes that do not result in an amino acid change are called synonymous changes. Synonymous changes can occur in the third base pair position of some (but not all) codons and the first base pair position of codons that code for the amino acid leucine and serine. Changes in the third base pair position that result in termination codons are rare particularly in mtDNA sequences where premature termination of the protein products required for energy metabolism may be lethal. The majority of nucleotide substitutions that occur in protein coding sequences are silent changes, rather than amino acid altering changes, preserving the overall structure of the amino acid sequence. Positional homology no longer becomes an obstacle to the correct alignment of protein coding sequences as the amino acid sequence can be used as benchmark for the alignment of sequences from different taxa.

Recent information regarding the rates of mtDNA divergence in poikilotherms indicates much slower rates of mutational change in cold blooded species such as fish, suggesting that the pattern of nucleotide divergence in fish mtDNA may not follow the same pattern as that of the mammalian homologue (Thomas and Beckenbach, 1987; Rand, 1993). The revelation of the unique patterns of nucleotide substitution observed in cold blooded species has challenged the accepted notions of mtDNA evolution and its position as the "molecule of choice" for population and evolutionary biology (Rand, 1993). Are the patterns of the accumulation of mutational change in fish species the same as those observed in mammals? The little information available comparing varying rates of mutational change within the mtDNA genomes of some fishes requires further investigation.

The Present Studies

The objectives of this thesis are to: 1) characterize the genetic differences between different region of mtDNA within, and between, three species of the genus *Salvelinus*; 2) to examine the genetic diversity of populations of *S. namaycush* and *O. mykiss* in Alberta; 3) to provide a description of the genetic structure of wild populations, stocked populations; and 4) assess the degree of possible introgression by hatchery strains into wild populations of these two species of Salmonidae.

This thesis is divided into three chapters: Chapter 1 gives the sequences and the phylogenetic relationships observed between three closely related members of the *Salvelinus*; the lake trout, *S. namaycush*, brook trout, *S. fontinalis*, and bull trout, *S. confluentus*. This chapter characterizes the genetic structure and relationships between these salmonid species and examines the interspecific levels of variation between these fish taxa. The chapter also examines the utility of using different regions of mtDNA, the non-coding control region and a region spanning two protein coding genes; the ATPase subunit 6 gene and the cytochrome oxidase subunit III gene, as genetic markers for identifying genetic differences at the stock and population levels. This chapter represents the first description of these sequenced regions of mtDNA for these members of the genus *Salvelinus* and provides essential sequence information for future monitoring of genetic changes.

In Chapter 2 the two protein coding regions described in Chapter 1 are examined by both RFLP analysis and DNA sequencing to investigate the mtDNA diversity of populations of the lake trout, *S. namaycush*, from Western-Canada. This chapter examines the genetic structure of lake trout populations that are currently recognized as either natural, introduced, or supplementally stocked in the recent past. Molecular characterization of populations of *S. namaycush* is accomplished in two parts. The first part examines individual fish from three lakes used as stock sources for the introduction of fish to Cold Lake, Alberta, to supplement the failing resident population of lake trout. Fish from the three donor populations are characterized genetically and compared to three different age classes within Cold Lake to quantify the degree to which introduced fish have integrated themselves within the Cold Lake population. The second part of this study further characterizes individuals from six other resident Alberta populations of lake trout, including native lake trout populations that have never been supplemented by stocking, lake trout populations that were introduced, and native populations of lake trout that have received supplemental stocking, to examine the levels of genetic differences between local populations that have not had contact with fish from the Cold Lake/donor lakes.

In Chapter 3 the same genetic markers examined in the first chapter and the RFLP methodology of Chapter 2 are used to examine the genetic diversity of a unique stock of *O. mykiss*, the Athabasca rainbow trout from Wampus Creek, a tributary of the MacLeod River. This strain of rainbow trout exhibits several unique morphological and genetic characteristics that suggest that the Athabasca Rainbow Trout diverged from any form of redband or coastal rainbow trout prior to the last glacial period, ~64,000 years ago. Trout from the Athabasca headwaters have similar coloration and pyloric caeca counts to other inland redband populations, but similar scale counts to coastal populations (Behnke, 1979; Bajkov, 1927). Moreover, these trout differ at several allozyme loci from both coastal rainbow populations and inland redband trout populations (Mayhood, 1992; Leon Carl *et al.*, Ontario Ministry of Natural Resources. Research Section, Fisheries Policy Branch in press). This unique stock of rainbow trout may represent a separate evolutionary group from all other stocks of rainbow trout. Individuals from this population were subjected to genetic analysis and compared to individuals from six sites within Alberta to examine the range of genetic differences between individuals from different localities. Individuals from the Alison Creek Brood Station, Alberta, and a hatchery strain of rainbow trout from Pennask Lake, British Columbia, are also compared to individuals from the Wampus Creek population in an attempt to identify genetic markers that would distinguish this unique stock of rainbow trout from introduced stocks of rainbow trout. The purpose of this part of the thesis is to identify molecular markers with which fish from the Wampus Creek population could be characterized, and to determine the degree to which hatchery propagated strains of rainbow trout have integrated with this unique endemic strain of rainbow trout.

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Chapter 1

Sequence Variation in the Mitochondrial DNA of the Lake Trout, *S. namaycush*, the Bull Trout, *S. confluentus*, and the Brook Trout, *S. fontinalis*.

Introduction

Detailed comparisons of mammalian mitochondrial DNA (mtDNA) have revealed several unique properties of mtDNA: a high degree of sequence variation relative to the nuclear genome; a small, highly conserved genome with regards to gene composition; no pseudogenes, introns, or highly repetitive DNA; a maternal mode of inheritance and a lack of recombination (Brown *et al.*, 1986; Foran *et al.*, 1988; Saccone *et al.*, 1991). These characteristics identified mtDNA as a valuable molecule for high resolution studies of evolutionary relationships between populations and closely related taxa (Ferris and Berg, 1987). Information regarding the rates of mutational change in different regions of mammalian mtDNA impies that the highest rates of change are found in the control region. Although the mitochondrial control region is the only major "non-coding" region of mtDNA (it does not code for a particular protein product) it does contain essential functional sites within its sequence. For example the site for the initiation of heavy-strand mtDNA replication, promoter sequences for both heavy and light strand RNA transcription, and several proposed termination-associated nucleotide sequences (TAS). In addition to these unique functional sites are several unique structural sites characteristic to the control region, such as several short conserved sequence blocks (CSB) of unknown purpose, and a centrally located polypyrimidine tract (Pyr) (Saccone *et al.*, 1991; Clayton, 1991; Digby *et al.*, 1992; Shedlock *et al.*, 1992).

Protein coding genes such as the ATPase subunit 6 (ATPase 6) gene and the cytochrome oxidase subunit III (COIII) gene are essential to oxidative metabolism, the primary role of the mitochondria within the cell, and exhibit intermediate rates of mutational change (Ferris and Berg, 1987; Cann *et al.*, 1984). The majority of sequence changes in protein coding genes favor nucleotide substitutions rather than insertions or deletions. This conserves the overall structure and composition of the amino acids required for the protein products of these genes (see Introduction). Moreover, nucleotide substitutions are silent (or synonymous), the majority (but not all) occurring in the third base pair position of codons and the first base pair position of codons coding for the amino acids leucine and serine. Amino-acid altering or non-synonymous substitutions in protein coding regions are less common with the majority occurring in either the first or second base pair position of codons (Li and Graur, 1991). Changes in the third base pair position that result in premature termination of the protein products are rare in the mtDNA as these mutations may interfere with energy metabolism and therefore be lethal .

Information regarding structural characteristics and evolutionary rates in non-mammalian vertebrate mtDNA indicates that the nature of mutational change in the mtDNA of different vertebrate taxa is not constant. Information regarding the sequence variation and structure of fish, turtle, and invertebrate mtDNA reveals some unique characteristics of mtDNA that differ drastically from the mammalian homologue: a fivefold decrease in rates of evolution, the occurrence of length overlaps between genes, and tandem duplication and repetition of sequences (Rand, 1994). The few studies of the sequence and structural characteristics of mtDNA in fish species (Schmidt and Gold, 1993; Beckenbach, 1990; Johansen *et al.*, 1990; Buroker *et al.*, 1990; Kocher *et al.*, 1989), and even fewer comparative examinations of the sequence variation and structure of genes in the mtDNA of salmonid fishes (Shedlock *et al.*, 1992; Digby *et al.*, 1992; Thomas and Beckenbach, 1989) indicate that the accelerated rates of mutation observed in the mtDNA of mammals may be an exception rather than the rule in the case of fishes. Clearly the dynamics of mutational change in groups such as the fishes requires further investigation.

In this chapter I describe the sequence composition, compare the levels of sequence divergence, and examine the phylogenetic relationships in two regions of DNA in the mitochondrial genome of three closely related species of the genus *Salvelinus*, the lake trout, *Salvelinus namaycush*, the bull trout, *Salvelinus confluentus*, and the brook trout, *Salvelinus fontinalis*. To fully understand the dynamics of mitochondrial evolution mutational changes in closely related taxa with low levels of sequence divergence must be examined to minimize the degree of "erasure" of initial changes by subsequent mutational changes. The members of *Salvelinus* examined here represents such a group. Two different regions of salmonid mtDNA were examined in this study. The noncoding control region was examined due to its potential to accumulate mutational change rapidly. A region spanning two protein coding genes, the ATPase-6/COIII genes, was examined to further characterize the dynamics of mutational change within salmonid mtDNA, and the evolutionary relationships between the three *Salvelinus* species.

Members of the salmonid genus *Salvelinus* are of recent origin (Pliocene or Pleistocene) and exhibit a little genetic divergence among taxa (Niel *et al.*, 1990; Behnke, 1980). The taxonomic relationships of species in this genus are unclear. Behnke (1980) divides the genus into three subgenera: *Cristovomer*, which includes *S. namaycush*; *Baione*, including *S. fontinalis* ; and *Salvelinus* which includes *S. confluentus*, *S. alpinus*, *S. malma*, and *S. leucomaenis*. Restriction digests of total mtDNA by Grewe *et al.*. (1990) supported this subdivision. Sequence comparisons of a nuclear locus, the internal transcribed spacer of ribosomal DNA (Peyete *et al.*, 1992), agreed with the pairing of *S. confluentus* with *S. leucomaenis*, but differed by pairing *S. fontinalis* with *S. namaycush*, and *S. alpinus* with *S. malma* as the most recently derived species pair. Stearly and Smith (1993), based on osteological comparisons, have grouped *S. namaycush* with *S. confluentus*, *S. fontinalis* with *S. leucomaenis*, and *S. alpinus* with *S. malma*. Thus the position of *S. confluentus* in the *Salvelinus* is unclear. The sequences obtained in this study were compared to published sequences obtained from other members of the *Salmonidae*. The phylogenetic relationships between *S. fontinalis*, *S. confluentus*, and *S. namaycush* were examined and compared to previously published morphological and molecular studies of the genus *Salvelinus*.

Materials and Methods

Polymerase chain reaction (PCR) amplified control region and ATPase-6/COIII products were each obtained from the genomic DNA of six separate individual specimens of *S. namaycush*, a single specimen of *S. fontinalis*, and a single specimen of *S. confluentus*. Oligonucleotide primers used to amplify the control region were patterned after conserved sequences from tRNA genes flanking the control region in rainbow trout (Shedlock *et al.* 1992; Digby *et al.*, 1992). External primer sequences flanking the control region were, t-Phe CST144 5' AGTGTTATGCTTTAGTTAAGCTAC 3', and t-Pro CST145 5' TAACTCCCAAAGCTAAGATTCTAA 3'. Primers used to amplify the ATPase-6/COIII region were patterned after the first 30 base pairs (bp) of sequences of this region in rainbow trout (Thomas and Beckenbach 1989). These primers proved to be unreliable and primers patterned after sequence data obtained from *S. namaycush* were constructed. External primer sequence for the ATPase 6 end was CST196 5' CGACCAATTTATGAGCCCCACATACC 3', and the COIII end was CST197 5' GAGACGTATAGGAAGAGCCATACGAC 3'. Subsequent internal primers were constructed from sequence data obtained from both the control region, and the ATPase-6/COIII region. Both strands of the control region were sequenced. All but 100 bp flanking the center of the ATPase-6/COIII genes were sequenced in both directions.

Genomic DNA used as a template was amplified by PCR in 100 µL reaction volumes containing 200 µM of each primer, 0.1 mM of each deoxynucleotide dATP, dTTP, dCTP and dGTP, 1x *Thermus aquaticus* (*Taq*) magnesium free polymerase buffer (Promega, Madison WI), 25 mM magnesium chloride, 5 u of *Taq* polymerase (Promega, Madison WI), enough sterile double distilled water to make a final volume 100 µL , and 10-500 ng of total genomic DNA. Amplifications were performed using a Perkin Elmer Cetus 480 DNA Thermocycler under the following conditions: one cycle of initial denaturation at 94^{o}C for 4 minutes, annealing at 56^{o}C for 30 seconds, primer extension (or synthesis) for 2 minutes at 72°C. The initial cycle was then followed by forty cycles of 94°C for 15 seconds, 54°C for 30 seconds, and 72°C for 2 minutes; and a final cycle of 94°C for 15 seconds, 54°C for 30 seconds, and 72°C for 10 minutes.

Amplified PCR products were purified by separation on a 1% agarose gel, the bands were excised, the isolated DNA was electroeluted, and the DNA resuspended at a final concentration from 50-500 ng/uL. Purified fragments were then sequenced using the Applied Biosystems DyeDeoxy Terminator Taq Cycle Sequencing Kit, and visualized on a Applied Biosystems 373A Automated DNA Sequencer. Sequences obtained from all three species were aligned against published control region, ATPase 6, and CO III sequences obtained from *Oncorhynchus mykiss* (Digby *et al.*, 1992; Peyete *et al.*, 1992; Thomas and Beckenbach, 1989).

Sequence analyses and alignments were performed using the Applied Biosystems SeqEd^{TM} 675 DNA Sequence Editor Version 1.00A. Deletions and insertions were scored on a presence-absence basis and included in further phylogenetic analysis along with all of the sequence data. Analysis of sequences were performed using MacClade 3.01 and cladistic analysis was performed using the Exhaustive search function of Swofford's (1985) Phylogenetic Analysis Using Parsimony 3.1.1 (PAUP). Bootstrap confidence intervals were estimated from 100 replicates. Trees were generated from each individual region of mtDNA examined (control region, ATPase 6 and COIII).

Results

The control region sequence presented in Figure 1-1 begins 8 bp into the non-coding region and stops at the end of the central T rich polypyrimidine tract of the control region. The first 8 bp were omitted from any analysis due to unresolved ambiguities in the sequence. Complete sequences of the control region were obtained from *S. namaycush* (five individuals), but were not fully resolved in the two other species in this study and were omitted from any further analysis. A total of 578 bp from the control region regions in all three species were examined. The sequences obtained from the 1380 bp ATPase-6/COIII region are presented in two separate figures (Figures 1-2 and 1-3). The inferred amino acid composition of these two protein coding genes are indicated above the *S. namaycush* sequence. Guanine was the least frequent nucleotide in all sequences examined (Table 1-1).

The majority of sequence changes observed in all regions examined were restricted to single base pair substitutions (Table 1-2). No insertions and deletions in the control region larger than 3 bp were detected. Transitions (a change of a purine to a purine: A or G; or, a change of a pyrimidine to a pyrimidine T or C) are a more common type of base substitution than transversions (all other nucleotide substitutions), and transversions become relatively more frequent between more distantly related taxa (McVeigh and Davidson, 1991). The ratios of transitions:transversions obtained were similar to values observed in other salmonid species for both the control region and other mtDNA protein coding genes (Shedlock, *et al.* 1992; McVeigh and Davidson, 1991). Ratios of transitions:transversions between each *Salvelinus* species and *Oncorhynchus mykiss* were similar. The frequencies of transversions in the two protein coding genes was to low to acurately indicate phylogenetic relationship, but the ratios of transitions to transversions for the control region indicated a closer relationship between *S. confluentus* and *S. namaycush.* A single base pair overlap was observed between the ATPase 6 and COIII genes. Comparisons of the *Salvelinus* sequences with published rainbow trout sequences

FIGURE 1-1. Nucleotide sequence of a 578 bp. segment from the 5' end of the control region obtained from the consensus sequence of five individual *S. namaycush*, a single *S. confluentus*, and a single *S. fontinalis*. The published sequence for *Oncorhynchus mykiss* was taken from Shedlock *et al.* (1992). Sequence begins 8 bp into the control region. Numbering begins at the origin of the control region and is indicated to the right of each row of sequence. Possible sites for the proposed termination associated signals (TAS) for control region synthesis and the central T rich polypyrimidine tract (Pyr) are overlined and labeled above the *S. namaycush* sequence. Sequence similarities between nucleic acids are indicated by a dot (.), and gaps resulting from deletions and insertions are indicated by a dash (-). Underlined nucleotides represent polymorphic sites within the five individual *S. namaycush* sequenced.

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                                                                                 .TAS.
S.namaycush       TAATATGTAC AACAATGAAC G-TTGTATC- TCAACAAATT AGTGTTATAA TACATCTATG  68
S.fontinalis      .......... .G....A..T .T.....C.. ...-...... ..G....... ..........
S.confluentus     .......... ..T......T A......... .......... .......... ..........
O.mykiss          AGC....... ...TG.A..T ....A..A.T .GT.-..CCC .A.......C ..........

                          .T AS.                                             .TAS
S.namaycush       TATAATATTA CATATTATGT ATTTACCCAT ATAT--AATA TCTGGGTGGT GAGTAGTACA  128
S.fontinalis      .......... ......G... .......... ...CAA.... ...CAA.... ..........
S.confluentus     .........G .......... .......... ....AC.... C...TA..A. ..........
O.mykiss          .......... .......... .......... ....A..-.. -...CA..-. ..........

                  -
S.namaycush       TCATATGTAT TATCAACATA -AGTGAATTT AAGCCCTCAT ATATCAGCAT AAACCCAAGA  188
S.fontinalis      .......... .......... .......... .......... .......... .T.......G
S.confluentus     .......... .......... .......... .......... .......... .........G
O.mykiss          .T........ .......... CG....T... ..C....... .C.......C ...T.....G

S.namaycush       TTTACAATAA GCTAAACACG TGATAATAAC CAACTAGGTT GTCTTAACCT -AGGTAATTG  248
S.fontinalis      ......-... .......... .......... .......... .......... ....C.....
S.confluentus     .G.G.G-... .......... .......... .......... ..T....... ....C.....
O.mykiss          ......T... ..C..-.... .......... ......A... ..T...A... G.T-......

S.namaycush       CTATATTAAT AAAACTCCAA CTAACACGGG CTCCGTCTTT ACCCACCAAC TTTCAGCATC  308
S.fontinalis      .A.......C .......... .......... .......... .......... ..........
S.confluentus     ....G..... .......... .......... .......... .......... ..........
O.mykiss          ......C... .......... .......... .......... .......... ..........

S.namaycush       AGTCCTACTT AATGTAGTAA GAACCGACCA ACGATTTATC AGTAGGCAT- ACTC-TTATT  368
S.fontinalis      .......... .......... .......... .......... .......C.A ....T.....
S.confluentus     .......... .......... .......... C......... ..C....... ..........
O.mykiss          .....GG... .......... .......... .......... G......... ..........

S.namaycush       GATGGTCAGG GGCACATATC GTATTAGGTA ACATCTCGTG AATTATTCCT GACATTTGGT  428
S.fontinalis      .....A.... ....G..... .......... .......... .......... .G........
S.confluentus     ......G.-. ....A..C.. .......... .......... ..C....... .G........
O.mykiss          .......... ....G..... .........C G......... .......... .G........

S.namaycush       TCCTAAGTCA AGGGCTATCC TTAAGAAACC AGCCCCTGAA AGCCGAATGT AAAGCATCTG  488
S.fontinalis      .........G .......... .......... .......... .......... ..........
S.confluentus     .........G .......... .......... .......... .......... ..........
O.mykiss          .......... .......... .......... .......... .......... ..........

S.namaycush       GTTAATGGTG TCAATCTTAT TGCCCGTTGA CCCACCAAGC CGGGCGTTCT CTTATATGCA  548
S.fontinalis      .......... .......... ........-. .......... .......... ..........
S.confluentus     .......... .......... ...T....-. .......... .......... ..........
O.mykiss          .......... .......... ........-. .......... .......... ..........

                       .____ ____Pyr___ _________.
S.namaycush       TAGGGTTCTC CTTTTTTTTT -TTTCCTTTC  578
S.fontinalis      .......... .......... ...C......
S.confluentus     .......... .......... ...C..-...
O.mykiss          .......... .......... C.........
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                Leu Gly Ile Pro Leu Ile Ala Val Ala Leu Thr Ile Pro Trp Ile Leu Phe Pro Thr Pro Ser Ala Arg 102
S. namaycush    CTA GGC ATC CCA CTT ATT GCC GTG GCA CTA ACC ATT CCC TGA ATT CTA TTC CCC ACC CCC TCC GCC CGT
S. fontinalis   ... ..A ... ... ... ... ... .AA .T. ... ..T ... ... ... ... ..C ... ... ... ... ... ... ...
S. confluentus  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ..T ... ... ... ... ...
O. mykiss       ... ..T ... ... ... ..C ... ..A .G. T.. ... C.C ..A ... ... ..T ... ..T ... ... ..T ... ..A

                Trp Leu Asn Asn Arg Leu Ile Thr Leu Gln Gly Trp Phe Ile Asn Arg Phe Thr Gln Gln Leu Leu Leu 171
S. namaycush    TGA CTA AAC AAT CGT CTA ATC ACT CTA CAA GGA TGG TTC ATC AAC CGA TTT ACT CAG CAA CTT CTT TTG
S. fontinalis   ... ... ... ... ... ... ..T ..C ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ...
S. confluentus  ... ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
O. mykiss       ... ... ... ..C ..C ... ..T ..C ..G ... ..G ... ... ... ... ... ... ..C ... ... ... ... ..A

                Pro Leu Asn Leu Gly Gly His Lys Trp Ala Val Leu Leu Thr Ser Leu Met Leu Phe Leu Ile Thr Leu 240
S. namaycush    CCC CTC AAC TTA GGG GGC CAT AAA TGA GCA GTT CTA CTA ACC TCC CTA ATA CTA TTC CTT ATT ACC CTA
S. fontinalis   ... ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ...
S. confluentus  ... ... ..T ... ..A ... ... ... ... ... ... ... ... ... ... ..G ... ..T ... ... ... ... ...
O. mykiss       ..G ..A ..T C.. ..C ..T ..C ..G ... ... .C. ... ... ..T ... ..C ... ... ..T ... ... ... ...

                Asn Met Leu Gly Leu Leu Pro Tyr Thr Phe Thr Pro Thr Thr Gln Leu Ser Leu Asn Met Gly Leu Ala 309
S. namaycush    AAT ATA CTG GGC CTA CTT CCA TAT ACA TTC ACC CCC ACC ACA CAG CTC TCT CTA AAT ATA GGC CTT GCA
S. fontinalis   ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ..A ... ..C ... ... ... ..T ... ...
S. confluentus  ... ... ... ... ... ... ..G ... ... ..T ... ... ... ... ... ... ..C ... ..C ... ... ... ...
O. mykiss       ... ... ..T ... ... ... ... ... ... ... ... ..G ... ... ... ... ..C ... ... ..G ... ..C ...

                Val Pro Leu Trp Leu Ala Thr Val Ile Ile Gly Met Arg Asn Gln Pro Thr Ala Ala Leu Gly His Leu 378
S. namaycush    GTA CCA CTA TGA CTA GCG ACA GTA ATT ATT GGC ATG CGG AAC CAA CCC ACC GCT GCC CTA GGT CAC CTC
S. fontinalis   ... ... ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ..C ..C ... ...
S. confluentus  ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ...
O. mykiss       ..C ... ..G ..G ..T ..T ... ... ... ..C ... ..A ..A ... ... ..T ..G ..C ... ..C ..C ..T T.A
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FIGURE 1-2. Nucleotide and inferred amino acid sequence of the ATPase subunit 6 gene of *S. namaycush*, *S. confluentus*, and *S. fontinalis*. The published sequence for *Oncorhynchus mykiss* was taken from Thomas and Beckenbach (1987). Sequence begins at the 33rd base pair from the origin of the gene and ends with the termination codon prior to the start of the cytochrome oxidase subunit III gene. Numbering begins at the origin of the gene and is indicated to the right of each row of sequence. Amino acid composition is indicated above the sequence of *S. namaycush*.. Sequence similarities between nucleic acids are indicated by a dot (.) and sequence changes in the *S. namaycush* sequence are underlined.

```
                 Leu Pro Glu Gly Thr Pro Val Pro Leu Ile Pro Val Leu Ile Ile Ile Glu Thr Ile Ser Leu Phe Ile 447
S. namaycush     TTG CCC GAA GGA ACC CCC GTC CCT TTA ATC CCG GTG CTT ATT ATT ATC GAA ACA ATT AGC CTT TTT ATC
S. fontinalis    ..A ... ..A ... ... ... ... ... ... ... ... ..A ... ..C ... ... ..G ... ... ... ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ..T
O. mykiss        ... ..T ... ... ... ... ..T ..A C.G ... ..A ..A ..G ..C ... ... ... ... ... ... ... ... ...

                 Arg Pro Leu Ala Leu Gly Val Arg Leu Thr Ala Asn Leu Thr Ala Gly His Leu Leu Ile Gln Leu Ile 516
S. namaycush     CGC CCC CTC GCC CTT GGT GTA CGA CTT ACA GCC AAT CTC ACG GCA GGC CAC CTT CTT ATT CAA CTG ATC
S. fontinalis    ... ... ..T ... ... ... ... ... ... ... ... ... ... ..A ..G ..T ... ... ... ... ... ... ..T
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ... ...
O. mykiss        ... ... --- ... ... ..C ... ... ... ... ... ... ... ..A ... ... ... .AA ..A ... --- --- ---

                 Ala Thr Ala Ala Phe Val Leu Leu Pro Leu Met Pro Thr Val Ala Ile Leu Thr Ser Ile Val Leu Phe 585
S. namaycush     GCC ACA GCA GCC TTC GTC CTC CTA CCC CTC ATA CCC ACA GTA GCG ATC TTA ACC TCT ATT GTC CTG TTC
S. fontinalis    ... ... ... ... ... ... ... T.. ... ... ... ..T ... ... ... ..T ... ... ... ... ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...
O. mykiss        ..T ... ... ... ..T ..T ..T ... ..T A.A ... ..T ... ... ..A ... C.. ..T ... ... ... ..C ..T

                 Leu Leu Thr Leu Leu Glu Ile Ala Val Ala Met Ile Gln Ala Tyr Val Phe Val Leu Leu Leu Ser Leu 654
S. namaycush     TTA CTT ACC CTT CTT GAA ATT GCC GTT GCT ATG ATC CAA GCC TAT GTC TTT GTT CTA CTA CTA AGC CTT
S. fontinalis    C.. ... ... ... ... ... ... ... ... ..C ..A ... ... ... ... ... ... ... ... ..C ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ..C ... ... ...
O. mykiss        C.. ..C ... ... ..C ... ..C ... ..A ..C ..G ..T ... ... ..C ..T ... ..C T.. ..C ... ... ..C

                 Tyr Leu Gln Glu Asn Val <O>
S. namaycush     TAT TTA CAA GAA AAC GTC TAA  675
S. fontinalis    ... ... ... ... ... ... ...
S. confluentus   ... ... ... ... ... ... ...
O. mykiss        ... ... ... ... ... ..T ...
```

(Continued from Figure 1-2)

```
                Met Ala His Gln Ala His Ala Tyr His Met Val Asp Pro Ser Pro Trp Pro Leu Thr Gly Ala Ile Ala
S. namaycush    ATG GCA CAC CAA GCA CAC GCA TAC CAC ATG GTT GAC CCA AGC CCC TGA CCT CTA ACC GGC GCA ATT GCC   743
S. fontinalis   ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S. confluentus  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
O. mykiss       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ...

                Ala Leu Leu Leu Thr Ser Gly Thr Ala Val Trp Phe His Phe His Ser Leu Thr Leu Leu Ala Met Gly
S. namaycush    GCC CTT TTA CTT ACA TCA GGC ACT GCA GTC TGA TTC CAC TTC CAC TCA CTT ACA CTA CTC GCC ATG GGA   812
S. fontinalis   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ...
S. confluentus  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ...
O. mykiss       ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ..G ..C ... ..T ..T A.. T.A ..T

                Asn Ile Leu Leu Leu Leu Thr Met Tyr Gln Trp Trp Arg Asp Ile Ile Arg Glu Gly Thr Phe Gln Gly
S. namaycush    AAT ATT CTA TTA CTT CTT ACC ATA TAT CAA TGA TGA CGA GAC ATT ATC CGA GAA GGT ACC TTC CAG GGG   881
S. fontinalis   ... ... ... ... ... ... ... ... ..C ... ... ... ... ..T ... ... ... ... ... ... ... ..A ...
S. confluentus  ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..C ... ... ..A ..A
O. mykiss       ..C ... ..C ... ... ..A ... ... ..C ... ... ..G ..G ..T ..C ... ... ... ... ... ..T ..A ..A

                His His Thr Pro Pro Val Gln Lys Gly Leu Arg Tyr Gly Met Ile Leu Phe Ile Thr Ser Glu Val Phe
S. namaycush    CAC CAC ACA CCC CCG GTT CAA AAA GGA CTA CGA TAC GGC ATA ATC TTA TTT ATT ACC TCC GAA GTA TTC   950
S. fontinalis   ... ... ... ... ..A ... ... ... ... ... ... ..T ... ... ..T ... ... ... ... ... ... ... ...
S. confluentus  ... ... ... ... ... ..C ... ... ..G ... ... ..T ... ... ... ... ... ... ... ... ... ... ...
O. mykiss       ... ... ..G ... ..A ..C ... ... ..G ... ... ..T ... ... ... ... ... ... ... ... ..G ... ...

                Phe Phe Leu Gly Phe Phe Trp Ala Phe Tyr His Ser Ser Leu Ala Pro Ser Pro Glu Leu Gly Gly Cys
S. namaycush    TTT TTC TTG GGC TTC TTC TGA GCT TTC TAC CAC TCT AGT TTA GCC CCC TCG CCT GAG TTA GGG GGC TGC   1019
S. fontinalis   ... ... ..A ... ... ... ... ..C ... ... ... ... ... ... ... ..T ..A ... ... ... ..T ... ...
S. confluentus  ... ... ..A ... ... ... ... ... ... ... ... ... ... ... ..A ... ..C ... ... ... ... ... ...
O. mykiss       ... ... ..A ..T ... ... ... ..C ... ... ... G.C ..C C.C ... ... A.A ... ..A ... ..A ..T ...
```

FIGURE 1-3. Nucleotide and inferred amino acid sequence of the cytochrome oxidase subunit III gene of three salmonid species, *S. namaycush*, *S. confluentus,* and *S. fontinalis*. The published sequence for *Oncorhynchus mykiss* was taken from Thomas and Beckenbach (1987). Sequence and numbering continue from the end of the ATPase 6 gene, and end 33 bp before the end of the COIII gene. There is a single bp overlap of the CoIII and the ATPase 6 genes; the first base pair of the Methionine codon of the COIII gene corresponds to the last bp of the termination codon of the ATPase gene. Sequence identity between the three species is indicated using a dot (.). Amino acid composition is indicated above the sequence of *S. namaycush*. Sequence changes in the *S. namaycush* sequence are underlined.

```
                 Trp Pro Pro Thr Gly Ile Ile Thr Leu Asp Pro Phe Glu Val Pro Leu Leu Asn Thr Ala Val Leu Leu
S. namaycush     TGG CCC CCC ACA GGC ATT ATT ACT CTT GAC CCA TTT GAA GTC CCA CTT CTA AAT ACT GCA GTC CTT CTA  1088
S. fontinalis    ..A ... ... ... ... ... ..C ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...
O. mykiss        ..A ... ... G.. ..T ... .C. ... ..A ... ..C ... ..G ..A ..C ... ..T ... ... ... ... ... ...

                 Ala Ser Gly Val Thr Val Thr Trp Ala His His Ser Ile Met Glu Gly Glu Arg Lys Gln Thr Val Gln
S. namaycush     GCA TCT GGT GTT ACC GTT ACA TGA GCC CAC CAT AGC ATT ATA GAG GGG GAG CGA AAA CAA ACC GTT CAA  1157
S. fontinalis    ... ..C ... ... ... ... ... ... ... ... ... ... ..C ... ..A ..A ... ..G ... ... ... ..C ...
S. confluentus   ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
O. mykiss        ... ... ... ..C ... ..A ... ... ... ... ..C ... ..C ... ..A ..T ..A ... ... ... ... A.. ...

                 Ala Leu Thr Leu Thr Ile Leu Leu Gly Phe Tyr Phe Thr Phe Leu Gln Gly Met Glu Tyr Tyr Glu Ala
S. namaycush     GCT CTT ACT CTC ACT ATC TTA TTA GGA TTC TAC TTC ACT TTC CTT CAA GGT ATA GAA TAT TAC GAA GCC  1226
S. fontinalis    ... ... ... ... ..C ... ... ..G ..G ... ..T ..T ... ... ... ... ... ..G ..G ... ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..G ...
O. mykiss        ... ... ... ... ... ... ... C.G ... ..T ... ... ... ... ..A ... ... ... ... ..C ... ... ...

                 Pro Phe Thr Ile Ala Asp Gly Val Tyr Gly Ser Thr Phe Phe Val Ala Thr Gly Phe His Gly Leu His  1295
S. namaycush     CCC TTT ACA ATC GCT GAC GGC GTA TAC GGC TCT ACT TTC TTT GTC GCC ACA GGA TTC CAC GGC CTA CAT
S. fontinalis    ... ... ... ..T ... ... ... ... ..T ... ... ..C ... ... ... ... ... ..G ... ... ... ... ...
S. confluentus   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
O. mykiss        ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ..T ... ... ..C

                 Val Ile Ile Gly Ser Thr Phe Leu Ala Ile Cys Leu Leu Arg Gln Ile Gln Tyr His Phe Thr Ser Glu
S. namaycush     GTA ATT ATT GGC TCT ACC TTT CTG GCC ATC TGC CTC CTA CGA CAA ATT CAA TAC CAT TTT ACA TCT GAA  1364
S. fontinalis    ... ..C ... ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ... ... ... ... ..C ...
S. confluentus   ... ... ... ... ... ... ... ..A ... ... ... ... ... ... ... ... ..G ... ... ... ... ... ...
O. mykiss        ... ... ... ... ... ... ... ... ... G.T ... ..T ... ... ... G.. ... ... ..C ... ... ... ...

                 His His Phe Gly Phe Glu Ala Ala Ala Trp Tyr Trp His Phe Val Asp
S. namaycush     CAT CAC TTC GGC TTT GAA GCT GCC GCC TGA TAT TGA CAC TTT GTA GAC  1412
S. fontinalis    ... ... ... ... ... ... ... ... ... ... ... ... ... ..C ..C ...
S. confluentus   ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
O. mykiss        ... ..T ..T ... ... ... ... ..T ... ... ... ... ... ... ... ...
```

(Continued from Figure 1-3)

revealed two deletions occurring in the ATPase 6 gene for all of the *Salvelinus* species examined. The first deletion was a three base pair deletion starting at 432 bp, that resulted in the loss of single leucine residue in all three species. The second deletion was a 9 base deletion starting at bp. 484, which caused the loss of three residues; glutamine, leucine, and isoleucine in all three of the *Salvelinus* species. Two nucleotide substitutions in the ATPase-6/COIII genes resulted in amino acid alterations between the *Salvelinus* species. The first was a change at bp 55 in the ATPase 6 sequence resulting in the substitution of a valine residue in *S. namaycush* and *S. confluentus* for a glutamic acid residue in *S. fontinalis*. The second amino acid altering change occurred in a single individual *S. namaycush* at bp 1272 in the COIII gene, that resulted in the substitution of an alanine residue with a threonine residue.

Sequence divergence among the control regions differed little among the three *Salvelinus* species, but differed more compared to the more distantly related *O. mykiss* (Table 1-3). Pairwise comparisons of the sequence divergence of the *Salvelinus* species examined and the published rainbow sequence revealed that within the *Salvelinus* the non-coding control region had slightly higher levels of sequence divergence than the two protein coding regions. Compared to the published rainbow sequence, the protein coding ATPase-6 gene had a higher level of sequence divergence than the COIII gene and the mitochondrial control region. In the case of the *Salvelinus* species the control region sequences revealed similar levels sequence divergence between all Salvelinus species. Between *S. confluentus* and *S. namaycush* sequence divergence in the protein coding genes was roughly half that observed between all other comparisons between *Salvelinus* species, and that observed in the noncoding control region.

Phylogenetic analysis of the individual regions of mtDNA resulted in phylogenetic trees of differing topologies between the non-coding control region and the two protein coding regions (Figure 1-4). Phylogenetic analysis, using the exhaustive search function of PAUP (Swoford 1985), of the noncoding sequences resulted in two most parsimonious

Table 1-1. Base compositions (%) of the control region and ATPase-6/COIII regions examined in three species: *S. namaycush* , *S. confluentus,* and *S. fontinalis.* The composition of each base: adenine, guanine, cytosine, and thymine, for each region examined is presented as a percentage of the total numbers of bases.

Species	Sequence	Base				Size (bp)
		A	C	G	T	
S. namaycush	ATPase 6	24.2	32.9	13.9	28.9	642
	CO III	24.7	29.4	16.8	29.2	738
	control region	30.9	21.5	15.7	31.9	578
S. confluentus	ATPase 6	23.8	33.3	14.8	28.8	642
	CO III	24.8	29.8	16.5	28.8	738
	control region	30.2	22.1	15.9	31.9	578
S. fontinalis	ATPase 6	24.5	31.9	13.3	30.2	642
	CO III	24.4	30.0	16.9	28.6	738
	control region	30.5	21.8	16.5	31.1	578

Table 1-2. Pairwise comparisons of the ratio of transitions:transversions and the total numbers of deletions of the control region, COIII, and ATPase 6 sequences between all taxa examined in this study and published sequence taken from Shedlock *et al.* (1992) and Thomas and Beckenbach (1989).

COIII - 738 bp.				
	O. mykiss	*S. fontinalis*	*S. confluentus*	*S. namaycush*
O. mykiss	-	62/19	56/18	57/17
S. fontinalis	0	-	40/5	34/4
S. confluentus	0	0	-	14/3
S. namaycush	0	0	0	-
ATPase 6 - 642 bp.				
O. mykiss	-	64/25	67/27	69/27
S. fontinalis	12	-	42/5	29/6
S. confluentus	12	0	-	14/5
S. namaycush	12	0	0	-
control region - 578 bp.				
O. mykiss	-	25/20	31/18	24/17
S. fontinalis	15	-	19/11	14/7
S. confluentus	13	6	-	19/6
S. namaycush	11	9	6	-

Note: The ratio of total transitions/transversions are presented in the upper portion of each section and total insertions and deletions are presented in the lower portion of each section.

Table 1-3. Percent sequence divergence of the control region and ATPase-6/COIII genes between all taxa examined in this study and published *O. mykiss* sequence was taken from Shedlock *et al.* (1992) and Thomas and Beckenbach (1989).

		COIII - 738 bp.		
	O. mykiss	*S. fontinalis*	*S. confluentus*	*S. namaycush* *
S. namaycush	10.1	5.1	2.5	0.7
S. confluentus	8.9	5.9	-	-
S. fontinalis	11.1	-	-	-
		ATPase 6 - 642 bp.		
S. namaycush	15.7	5.2	2.8	0.8
S. confluentus	15.9	7.0	-	-
S. fontinalis	15.1	-	-	-
		control region - 578 bp.		
S. namaycush	8.9	5.8	5.4	1.2
S. confluentus	11.2	6.5	-	-
S. fontinalis	11.1	-	-	-

*calculated from the five *S. namaycush* individuals sequenced.

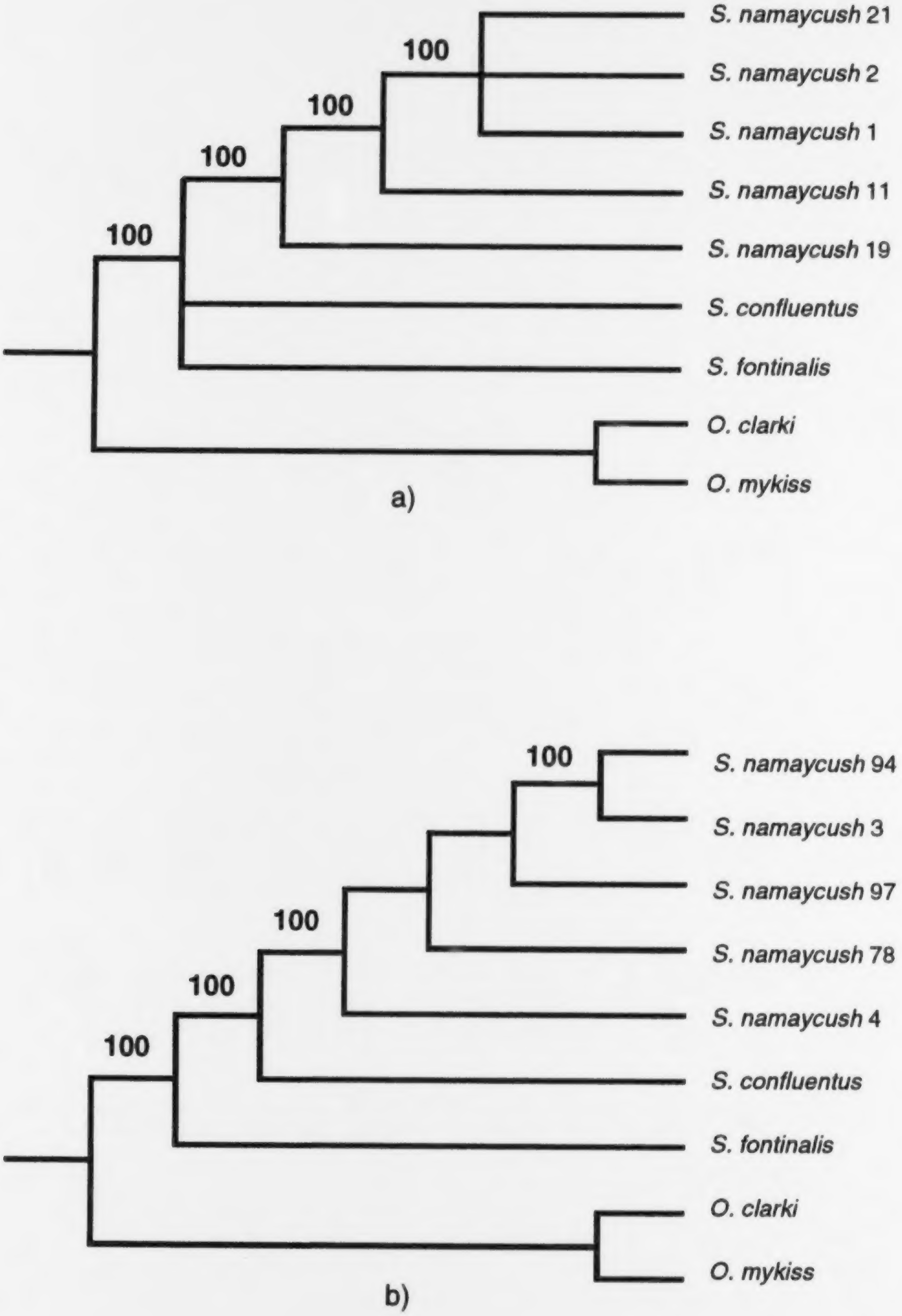
trees of 130 steps. Phylogenetic analysis of the protein coding regions resulted in four most parsimonious trees with 289 steps. Consensus trees were calculated for all trees in both the noncoding and protein coding regions (Figure 1-4). Two *Oncorhynchus* species, the rainbow trout, *O. mykiss*, and the cutthroat trout, *O. clarki*, were used as outgroups in the phylogenetic analysis. These two species belong to a sister genus to the *Salvelinus* and the complete sequences from all the mtDNA regions examined in this study were available for comparisons. Bootstrap of the consensus trees using the Branch and Bound search function with 100 replicates revealed that the control region failed to adequately resolve the position of *S. confluentus* and *S. fontinalis* with respect to *S. namaycush.* Bootstrap of the sequence obtained from the protein coding regions separates *S. confluentus* and *S. namaycush* from *S. fontinalis* with a greater degree of consistency (Figure 1-6).

Discussion

The distribution of nucleotides within all three regions is consistent with those observed in other organisms. Mitochondrial genes that are encoded on the heavy strand tend to have a low frequency of guanine, and in the third codon position of protein coding genes there tends to be a bias against both thymine and guanine (Meyer, 1994). Structural characteristics for the regions of mtDNA sequenced in this chapter are similar to those observed in other members of the Salmonidae. Possible sites for proposed termination associated sequences (TAS) and the central T rich polypyrimidine tract of the non-coding control region in *S. namaycush, S. confluentus*, and *S. fontinalis*, approximate closely to the sites proposed by Shedlock *et al.* (1992) and Digby *et al.* (1992) for rainbow trout, *O. mykiss.* The amino acid compositions of the ATPase 6 and COIII genes are similar amongst the three species of *Salvelinus* examined.

The distribution of steps (changes per site) across all regions sequenced, reconstructed from the two phylogenetic trees presented in Figure 1-4, revealed that there is a single conserved region around the start of the COIII gene (approximately from sites 640

Figure 1-4. Consensus trees generated from a) two most parsimonious trees (130 steps) obtained from the noncoding control sequences and, b) four most parsimonious trees (289 steps) obtained from the protein coding ATPase-6/COIII sequences. Sequences were obtained from two seperate groups of five *S. namaycush*, a single *S. confluentus*, and a single *S. fontinalis*. The published sequences of *O. mykiss* and *O. clarki* were used as "outgroups," taken from Shedlock *et al.* (1992) (control region) and Thomas and Beckenbach (1987) (ATPase-COIII). Trees were calculated using the Exhaustive search function of PAUP with a 50% majority rule (Swofford, 1993). The frequencies (≥50%) of each branch point from the total number of trees examined are indicated at the origin of each branch. Frequencies for branches below 50% were omitted and were considered interchangeable at those branch points.


100
100
100
100
S. namaycush 21
S. namaycush 2
S. namaycush 1
S. namaycush 11
S. namaycush 19
S. confluentus
S. fontinalis
O. clarki
O. mykiss
a)
100
100
100
100
S. namaycush 94
S. namaycush 3
S. namaycush 97
S. namaycush 78
S. namaycush 4
S. confluentus
S. fontinalis
O. clarki
O. mykiss
b)

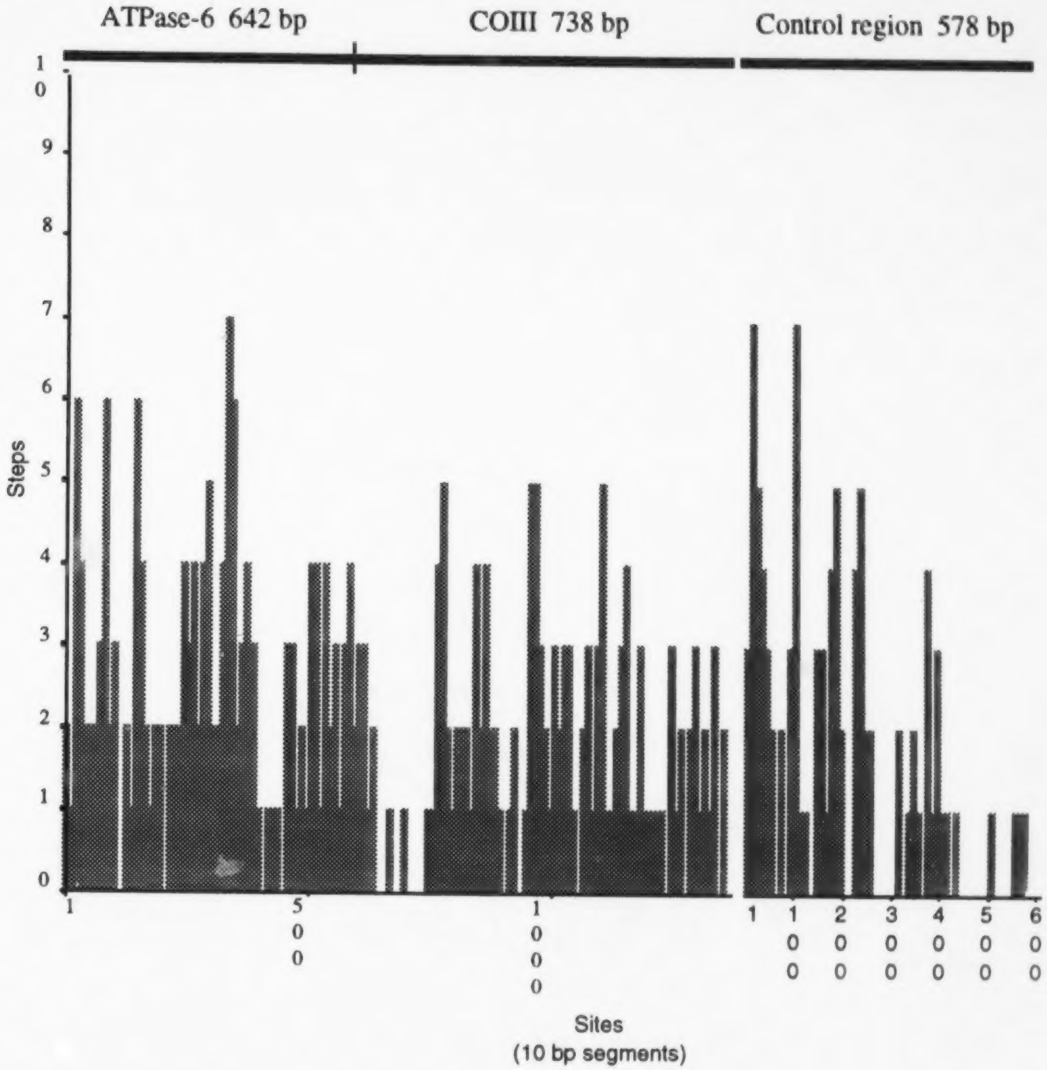


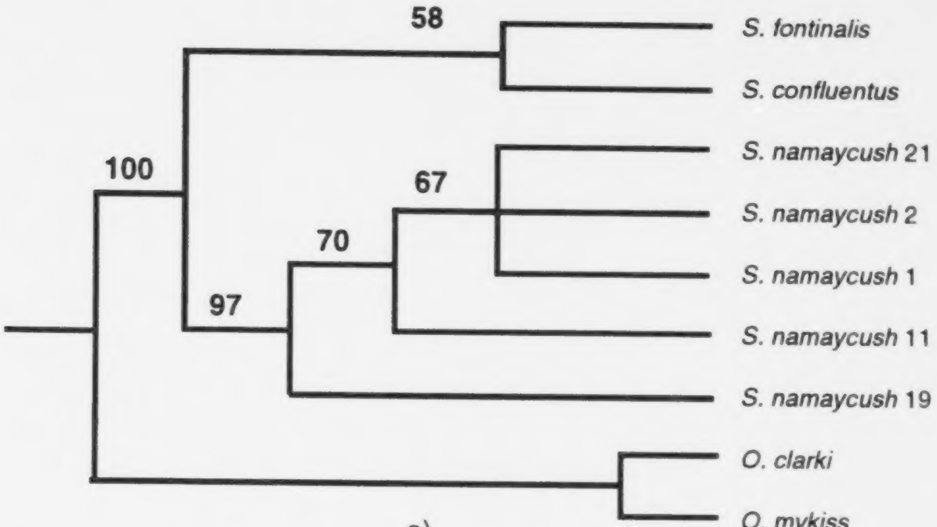
Figure 1-5. Distribution of variation across the all regions sequenced in *S. namaycush*, *S. confluentus*, *S. fontinalis*, and published outgroup sequences taken from *O. mykiss* and *O. clarki*, Shedlock et al. (1992) and Thomas and Beckenbach (1987), for the control region and ATPase-6/COIII sequences respectively. The number of steps (vertical axis) per site (horizontal axis) were reconstructed from the consensus trees generated in figure 1-4. Sites are grouped into 10 bp segments. The position and size of each of the regions sequenced and are indicated above the figure.

to 750). Two large conserved regions occur in the latter half of the control region sequence (one at approximately bp 250 to 350, and the second from 410 to 550 bp). Overall the number of steps (or changes) in the control region sequences are similar to the number of steps (changes) in the two protein coding genes. This suggests a similar numbers of mutational changes between the non-coding region and these two protein coding genes in fish mtDNA. The noncoding control region appears to be under some functional or structural constraint with regards to the accumulation of mutational change. This is contrary to accepted notions about the dynamics of change in the control region derived from mammalian sequences. The above observations should be interpreted with some caution as only the 5' end of the *Salvelinus* control region was fully resolved for all of the species in this analysis. Previous studies suggest that the highest levels of sequence divergence are localized to the 3' end of the control region, as much as 5 times that of the rest of the control region, (Shedlock *et al.*, 1992). The degrees of sequence divergence between all three regions of mtDNA were similar for all of the taxa examined. *S. namaycush* and *S. confluentus* exhibited the least amount of sequence divergence (Table 1-3), suggesting a closer genetic relationship between these two species than has been recognized previously.

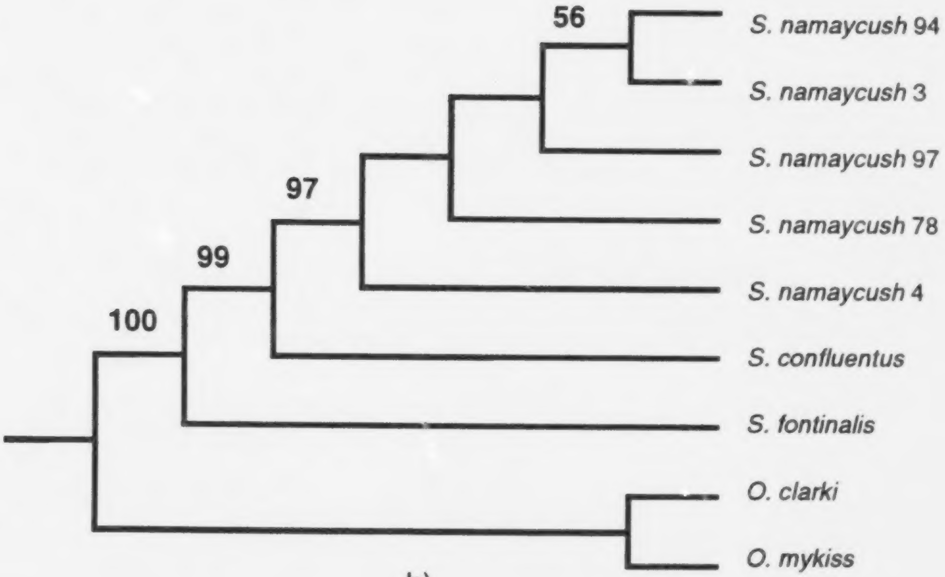
Phylogenetic analysis of the control region and the two protein coding regions resolved the evolutionary relationships between the three *Salvelinus* species with differing levels of consistency (Figure 1-6). Analysis of the control region sequences failed to adequately resolve the relationships between the three *Salvelinus* species. Consistency index in a total of 100 bootstrap replicates was 58 for the division of *S. fontinalis* and *S. confluentus* from *S. namaycush.* Reasons for the ambiguity of the control region sequences may be due to the difficulty associated with the alignment, and therefore identification of "positional homology," of noncoding regions (Meyer, 1994). Analysis using the protein coding regions clearly favors the pairing of *S. namaycush* with *S. confluentus* rather than with *S. fontinalis*, consistency index of 99 (Figure 1-6).

Taxonomy based upon morphological data divides the genus *Salvelinus* into three subgenera with *S. namaycush*, *S. fontinalis*, and *S. alpinus* (*S. confluentus* is grouped with *S. alpinus*) as the type representatives of the three subgenera (Behnke, 1989). This subdivision was supported by RFLP analysis of mtDNA by Grewe *et al.* (1990). The analysis presented here disagrees with these findings. Reasons for the discrepancy may be a result of the enhanced resolving power of sequence analysis. Restriction analysis is limited to those sites associated with the recognition sequences of the restriction enzymes employed and may not be sufficient to resolve the branching order of these closely related species (Wilson, 1987). Sequence analysis of a nuclear internal transcribed spacer of ribosomal DNA by Pleyete *et al.* (1992) did not divide these species into the above three subgenera, but did group *S. namaycush* with *S. fontinalis* separate from *S. confluentus*. The placement of *S. confluentus* within the genus *Salvelinus* varies in a large number of studies examining both morphological (Stearly and Smith 1993; Behnke 1989; Cavender 1980) and molecular data (Crane, 1992; Pleyete *et al.*, 1992; Grewe *et al.*, 1992). The sequence information presented here contradict Behnke's (1989) morphological data and the RFLP data of Grewe *et al.* (1992), but concur with an osteological study of the Salmonidae by Stearly and Smith (1993). In their analysis of the comparative anatomy of skeletal features among both living and fossil members of the Salmonidae Stearly and Smith (1993) group *S. namaycush* with *S. confluentus*, and position *S. fontinalis* as the sister group to the other two species. The difference in the evolutionary positioning of these three species between the mtDNA sequences obtained in this study and the nuclear sequence data of Pleyete *et al.* (1992) suggests the possible occurrence of an ancestral hybridization event during the history of divergence of these three species.

Figure 1-6. Bootstrap analysis of the consensus trees obtained from the a) noncoding control region, and b) the protein coding ATPase-6/COIII genes (see Figure 1-4). Bootstrap analysis was performed using a 50% majority rule and the Branch and Bound option of PAUP (Swofford, 1993). Numbers indicate a the frequency of each branch point (≥50%) from a random construction of trees from a total of 100 bootstrap replicates.


58
100
67
70
97
S. fontinalis
S. confluentus
S. namaycush 21
S. namaycush 2
S. namaycush 1
S. namaycush 11
S. namaycush 19
O. clarki
O. mykiss

a)


56
97
99
100
S. namaycush 94
S. namaycush 3
S. namaycush 97
S. namaycush 78
S. namaycush 4
S. confluentus
S. fontinalis
O. clarki
O. mykiss

b)

Summary

I examined three regions of mtDNA which exhibited features similar to those observed in other salmonid species. Levels of sequence divergence between the non-coding control region and the two protein coding regions, ATPase 6 gene and COIII gene, were comparable indicating similar rates of evolution between the non-coding region and protein coding regions in the mtDNA of these species of fish. Phylogenetic comparisons of the combined sequence information supported this observation. The control region exhibited only a slight increase in the degree of genetic variation when compared to the two mtDNA protein coding regions; not the 3 to 5 fold increase in accumulation of genetic variation expected from published mammalian data. Given the similar rates of sequence divergence observed between the non-coding and protein coding regions of the mtDNA, and the ambiguities over positional homology in the alignment of non-coding regions, protein coding regions should be favored over non-coding regions for phylogenetic and population studies of fish species.

Phylogenetic analysis of the sequence data from the three *Salvelinus* species did not agree with published phylogentic analysis of sequence data obtain from nuclear DNA and RFLP analysis of the mtDNA of members of the genus *Salvelinus*. The data presented here favored the grouping of lake trout with bull trout, over the traditional pairing with brook trout. These findings support recent osteological studies of these three species. The scope of this examination of the phylogenetic relationships between members of the genus *Salvelinus* is limited. Further examination of the mtDNA of members of all the Salmonidae is required to clarify the relationships within this diverse group of fish species.

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Chapter 2

Variation in Mitochondrial DNA (mtDNA) in Populations of Lake Trout, *Salvelinus namaycush*, in West-Central Canada

Introduction

Lake trout, *Salvelinus namaycush*, have a broad native distribution across most of Canada, extending from Alaska to Labrador; including a large number of the northern lakes in all of the prairie provinces; across several Arctic islands; northern British Columbia, and from the Great Lakes to Nova Scotia (Nelson and Paetz, 1992). In Alberta this species is believed to be native to the Slave, Peace, Beaver river drainage, and the North and South Saskatchewan river drainage (Nelson and Paetz, 1992; Mayhood, 1992). Superimposed upon the native distribution of this species is the recent transfer and introduction of *S. namaycush* from varying stocks and geographic locations. During the first half of this century the introduction of fish to western Canada from as far away as the Great Lakes was not uncommon and populations of native lake trout have undergone extensive transfer between populations within Alberta (Mayhood, 1992; Dave Donald, Inland Waters Directorate, Regina, Sask., pers. com.). The consequences of these historical introductions on the genetic diversity of natural populations of lake trout are unclear, creating a need for the genetic characterization and identification of lake trout populations across their existing geographic distributions.

Prior studies of the genetics of fish populations utilized allozyme electrophoresis for the genetic characterization of fish populations and stocks. Although protein electrophoresis has provided valuable insights into the genetics of both natural and hatchery propagated fish populations (Ihssen *et al.*, 1988; Ryman and Stahl, 1980) these past attempts have been "constrained because many species lack sufficient genetic divergence at polymorphic loci to permit population discrimination" (Billington and Hebert, 1991). Present day methods of directly examining genetic variation at the DNA level have greatly enhanced the resolving power and efficiency of the genetic characterization of fish populations. One type of DNA that has been widely adopted for population-level genetic studies in fish is mitochondrial DNA (mtDNA). Analysis of variation in mtDNA has been successfully used to relate phylogenetic patterns to geographic distributions of fish species

(Guiffra *et al.*, 1994; Fajen and Breden, 1992), the examination of evolutionary relationships among closely related taxa (Grewe *et al.*, 1990), and the detection of hybridization and introgression (Wilson and Hebert, 1993).

The majority of previously published studies of *S. namaycush* have examined the evolutionary relationships of *S. namaycush* to other members in the genus *Salvelinus* (Peyete, 1992; Grewe *et al.*, 1990; Clayton and Ihssen, 1980). Examinations of mtDNA diversity in populations of various fish species have indicated a few common haplotypes with a larger number of rare haplotypes that are the "mutational derivatives" of the more common types (Billington and Hebert, 1991). The restriction site variation of total mtDNA among populations of lake trout has a similar pattern of genetic variation. A total of 18 polymorphic loci were used to identify different strains of fish (Grewe and Hebert, 1988). A recent study of the mtDNA variation among lake trout strains in Lake Ontario using the variable restriction site loci identified by Grewe and Hebert (1988) revealed a total of seven mtDNA haplotypes distributed amongst the six hatchery strains examined (Grewe *et al.*, 1993). Total mtDNA digests of a total of 492 fish failed to assign individual fish to one of the six hatchery strains examined, but significant differences did exist between the frequencies of the seven haplotypes in the six strains. These restriction fragment length polymorphism (RFLP) examinations of mtDNA sequence variation among populations of lake trout suggests that northern species such as the lake trout exhibit some of the lowest levels of genetic divergence among fish species (Grewe and Hebert, 1988).

Restriction analysis of total mtDNA offers a limited glimpse at the amount of genetic variation that exist between groups with low levels of genetic divergence. The development of the polymerase chain reaction (PCR) allows specific regions of DNA to be isolated and amplified, facilitating the detection of genetic differences using both RFLP analysis and DNA sequencing (Beckenbach, 1991). Direct DNA sequencing provides the greatest resolution for the detection of genetic differences between individuals and populations, and the greatest amount of information for clarifying phylogenetic relationships between organisms. For species such as lake trout, analysis of mtDNA by restriction enzymes may not be sufficient to resolve the genetic relationships at the population level. Sequencing of DNA is required to adequately examine the genetic relationships between different populations.

The present study utilized PCR, restriction fragment polymorphism analysis (RFLP), and direct DNA sequencing to examine variation in mtDNA among populations of lake trout from west-central Canada. The sequence variation of homologous regions of *S namaycush* mtDNA encompassing two protein coding genes, the ATPase subunit 6 gene and the cytochrome oxidase subunit III gene, were initially examined using four base restriction enzymes to characterize different mtDNA types (haplotypes). Phylogenetic relationships between different haplotypes from different geographic locations were then examined using DNA sequencing.

Lake trout from a total of ten lake populations (Figure 2-1) with varying stocking histories (Table 2-1) were examined in this study. Included in the ten populations examined were fish from three lakes used as donor lakes, or stock sources, for supplemental stocking of lake trout to Cold Lake, Alberta. Comparisons of the donor lakes' fish and three different age classes, or resident sub-populations, within Cold Lake were made to examine the stock composition of lake trout in Cold Lake, and the degree of recruitment of introduced fish to the resident population. The six other resident Alberta populations of lake trout included: 1) populations that are endemic and have not received supplemental stocking; 2) populations that are endemic but have received supplemental stocking, and; 3) populations that have been introduced. Individuals from these populations were compared to individuals from the Cold Lake-donor lakes study to determine if the genetic types present in Cold Lake and the donor populations are commonly distributed across populations in Alberta.

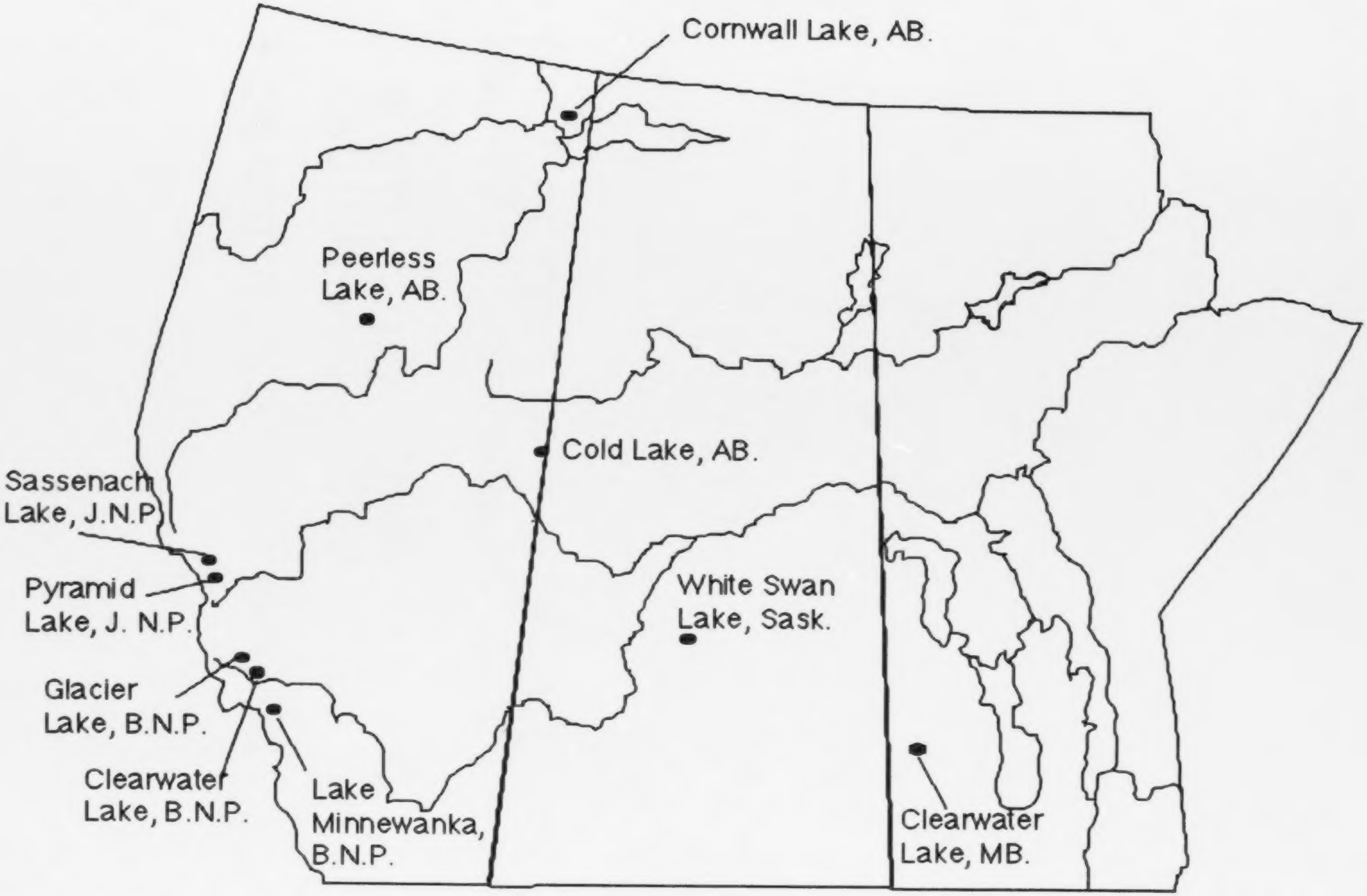

Cornwall Lake, AB.
Peerless Lake, AB.
Cold Lake, AB.
Sassenach Lake, J.N.P.
Pyramid Lake, J. N.P.
White Swan Lake, Sask.
Glacier Lake, B.N.P.
Clearwater Lake, B.N.P.
Lake Minnewanka, B.N.P.
Clearwater Lake, MB.

Figure 2-1. The location of all ten lake trout populations examined in Chapter 2.

Table 2-1. Status and stocking history of the ten lake trout populations from west central Canada (J.N.P. = Jasper National Park , and B.N.P. = Banff National Park). The sample sizes of fish from each population are indicated in the last column. The following abbreviations represent the following fish species: LT = lake trout; RT = rainbow trout; CT = coho salmon; BT = brook trout; CT = cutthroat trout; SPL = splake.

Lake	Prov.	Depth (m)	Status of LT	sp. stocked	N
Cold	AB.	99.1	native	LT,RT,CS*	43
Clearwater	MAN.	-	native	none	23
White Swan	SASK.	-	native	none	26
Cornwall	AB.	-	native	none	8
Pyramid	AB., J.N.P.	19.0	native	LT,RT,BT	9
Sassenach	AB.,J.N.P.	16.0	introduced	none	15
Glacier	AB.,B.N.P.	38.0	native	none	15
Clearwater	AB.,B.N.P.	-	native	none	4
Minnewanka	AB.,B.N.P.	99.1	native	LT,RT,BT, CT,SPL	19
Peerless	AB.	35.4	native	none	11

Study Lakes

The following is a brief description of the lakes and the resident populations of lake trout. Where possible information was obtained from both published and unpublished sources, National Parks personnel and stocking records. Detailed information regarding the stocking of lake trout into Cold Lake was provided by Alberta Fish and Wildlife (McCart and Jacobson, 1992).

Cold Lake, AB- This lake supports one of the most diverse fish faunas among Alberta lakes (Mitchel and Prepas, 1990). There has been an active lake trout fishery in Cold Lake since the early 1920's, but the fishery had diminished by the early 1940's and spawning surveys in the late 1980's failed to find many mature lake trout (McCart and Jacobson, 1992). Lake trout from several stock sources were introduced as early as 1965 to supplement the failing endemic population. The degree to which these introduced fish have integrated with the resident fish is unclear and the actual stock component of the resident fish in Cold Lake is unknown. Historical stocking of lake trout to Cold Lake has occurred from several source lakes: Cornwall Lake, Alberta, White Swan Lake, Saskatchewan, Clearwater Lake, Manitoba, and Lac la Ronge, Saskatchewan (see Table 2-2, for a summary).

Clearwater Lake, Man.- Clearwater Lake, Riding Mountain National Park, Manitoba, is home to an endemic population of lake trout. No lake trout have been introduced into Clearwater Lake. It has served as a native source stock for fisheries stocking efforts both within and outside of Manitoba (G. Sterling, Alberta Fish and Wildlife, personal. communication). Fish from Clearwater Lake were introduced into Cold Lake as early as 1974 (McCart and Jacobson, 1992).

White Swan Lake, Sask.- Supports an endemic population of lake trout. No lake trout have been stocked in White Swan Lake. Fish from this lake have been introduced to smaller lakes within Saskatchewan (Cram, 1977).

Table 2-2. The stocking history of Cold Lake, Alberta, 1965 - 1987. The figures were modified from McCart and Jacobson (1992). The following dates represent recorded stocking events and the origins of introduced fish.

Year	Mo.	No. Stocked	Egg Source	Stocking Rate (no./ha)
1965	June	54,000	Lac La Ronge, SK	1.5
1967	Sept.	69,920	Tulip Lake,AB; Whiteswan Lake SK	2.0
1968	June	121,535	Whiteswan Lake, AB; Whiteshell Lake, MB	3.5
1970	Sept.	76,950	Whiteswan Lake, AB	2.2
1971	Sept.	65,700	Whiteswan Lake, AB	1.9
1974	Aug.	91,000	Clearwater Lake, MB; Peerless Lakes, AB	2.6
1975	July	97,500	Clearwater Lake, MB	2.8
1976	July	319,092	Clearwater Lake, MB	9.1
1977	July	19,500	Clearwater Lake, MB	unknown
1978	July	95,040	Clearwater Lake, MB; Cornwall Lake, AB	2.7
1979	June	159,355	Cornwall Lake, AB	4.6
1980	July	90,735	Cornwall Lake, AB	2.6
1981	May	208,321	Cornwall Lake, AB	6.0
1982	June	159,980	Cornwall Lake, AB	4.6
1983	July	150,000	Cornwall Lake, AB	4.3
1986	June	108,580	Clearwater Lake, MB	3.1
1987	June	157,336	Cornwall Lake, AB	4.5
Total Stocked		2,025,044		

Cornwall Lake, AB.- Cornwall Lake is a small northern Alberta lake, located on the Canadian Shield. The lake trout population is believed to be endemic to the lake, as records indicate that it has never been stocked (G. Sterling personal. communication). Due to the inaccessible northern location it is unlikely that the present lake trout population was established from unrecorded stocking efforts. It is more likely that the resident lake trout population invaded the lake from the nearby Athabasca Lake population.

Peerless Lake, AB.- McIntyre (1990) reviews the physical and biological characteristics of Peerless Lake. Although fish may have entered this lake from the Peace River system by adjacent Graham and Trout Lakes, the population of lake trout is believed to be endemic and there is no record of stocking of lake trout.

Pyramid Lake, Jasper National Park- Mayhood (1992) reviews the history and status of the lake trout population of both Pyramid and Sassenach lakes. Pyramid Lake is one of only ten lakes in the Rockies that once contained a native species of lake trout. Records of fish spawning and tourist catches of large lake trout from Pyramid Lake are as early as 1910 and stocking of fish prior to the turn of the century is unlikely (Mayhood, 1992; Ward, 1974). Early records indicate that Pyramid Lake was not stocked prior to 1917, but lake trout have subsequently been stocked at least 51 times with fish from as far away as the Great Lakes (Mayhood, 1992). The native stock of fish in Pyramid Lake is likely to have undergone extensive hybridization with introduced stocks and it is likely that the native Pyramid Lake stock of fish no longer exists.

Sassenach Lake, Jasper National Park- Outside of Pyramid Lake no other native population of lake trout is believed to have existed within Jasper National Park. The present day population of lake trout in Sassenach Lake is considered to be the result of unrecorded introductions of fish to the lake (Donald and Alger, 1992; Mayhood, 1992).

Clearwater Lake, Banff National Park- Clearwater Lake supports an indigenous population of lake trout (Donald and Alger, 1992). Banff National Park stocking records indicate that this lake was never stocked with lake trout (T. Hurd, Park Warden, unpublished report). It is possible that the lake was invaded by fish from the nearby Glacier or Swan Lake populations. Unfortunately the stocking records for Banff National Park are less complete than those of Jasper National Park (Schindler *et al.* in press, Hurd, in prep), and the possibility that an unrecorded introduction or invasion of Clearwater Lake by introduced fish cannot be dismissed.

Glacier Lake, Banff National Park- Glacier Lake supports a native population of lake trout possibly resulting from invasion from the nearby Swan Lake (Nelson and Paetz, 1992; Donald and Alger, 1992). Again the park stocking records show that this lake had never been stocked with fish of any type (T. Hurd, Park Warden, unpublished report; D. Donald pers comm.), but the possibility of unrecorded stocking cannot be completely ruled out.

Lake Minnewanka, Banff National Park- This lake is one of the largest lakes in the Canadian Rockies and is subject to a high degree of recreational use. The lake supports a number of fish species, including a large native population of lake trout. A large variety of fish species have been introduced to Lake Minnewanka, with lake trout stocked as early as 1914.

Materials and Methods

Sample Collection and Isolation of DNA

Lake trout samples were obtained from a variety of sources. Fish from Cold Lake and the three donor lakes; Cornwall, AB, Clearwater, MB, and White Swan, SASK. were provided by George Sterling of Alberta fish and Wildlife, and both the Saskatchewan and Manitoba Fisheries branches. Lake trout from Clearwater Lake and Glacier Lake, Banff National Park, were supplied by Dave Donald, of the Inland Waters Directorate, Regina, Saskatchewan. Lake trout from Lake Minnewanka, B.N.P., Pyramid Lake and Sassenach Lake, Jasper National Park were supplied by the Canadian Parks Service. Fish from Peerless Lake were also supplied by Alberta Fish and Wildlife. Fish from wild populations were collected using gill nets or collected by the commercial fisheries.

Following collection, fish were delivered to the University of Alberta DNA Repository and samples of 2-5 grams of hypaxial muscle were catalogued and stored at -70^{o}C for future research. Further tissue samples of 0.5 grams were frozen in liquid nitrogen, homogenized by hand with a mortar and pestle, and resuspended in 1 ml of 0.15 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM disodium EDTA (ACK) lysis buffer at 4^{o}C. Total genomic DNA was then extracted using an Applied Biosystems Genepure 341 Automated DNA Extractor. Total genomic DNA was resuspended in a Tris EDTA buffer and stored at -20^{o}C for subsequent genetic analysis.

Primers and PCR Conditions

The ~1400 bp region spanning the ATPase 6 and COIII genes was amplified using oligonucleotide primers designed from sequences provided by Thomas and Beckenbach (1989). Published sequences from 6 salmonid species were used to construct primers CST 55 and CST 54 (Table 2-3). This primer set proved to be unreliable for the amplification of PCR product from *S. namaycush*, and a second set of primers over the same region was constructed from sequence data obtained from *S. namaycush* (see Chapter 1). Target

Table 2-3. Oligonucleotide primers used to amplify PCR product from *S. namaycush*. Primers 1 - 4 were used to amplify the entire fragment containing both the ATPase 6 - COIII genes, primers 5 - 8 were used to sequence the internal regions in these two genes.

Primer	Sequence (5' - 3')	Source
1. CST 54	GTAAATAGAGACGTATAGGAAGAG	*Oncorhynchus* spp.*
2. CST 55	GCTTCTTCGACCAATTTATGAGCC	*Oncorhynchus* spp.*
3. CST 196	CGACCAATTTATGAGCCCCACATACC	*Salvelinus namaycush* †
4. CST 197	GAGACGTATAGGAAGAGCCATACGAC	*S. namaycush* †
5. CST 277	CCACTATGACTAGCGACAG	*S. namaycush* †
6. CST 278	GGTGGGCTCATGTAACGGTAAGACC	*S. namaycush* †
7. CST 341	CCACAGGCATTATTACTC	*S. namaycush* †
8. CST 342	GCAGCGGTGGGTTGGTTC	*S. namaycush* †

* Sequences were taken from Thomas and Beckenbach (1989)

† Sequences were obtained from this study and are presented in Vitic *et al.* (unpublished data)

regions of DNA were amplified by PCR in 100 μL reaction volumes. Reaction mixtures were adjusted to optimize PCR product yields and contained 200 μM of each primer, 0.1 mM of each deoxynucleotide dATP, dTTP, dCTP and dGTP, 1x *Thermus aquaticus* (*Taq*) magnesium free polymerase buffer (Promega, Madison WI), 2.5 μM, 5 U of *Taq* polymerase (Promega, Madison WI), 10-500 ng of total genomic DNA, and enough sterile double distilled water to make a final volume of 100 μL respectively.

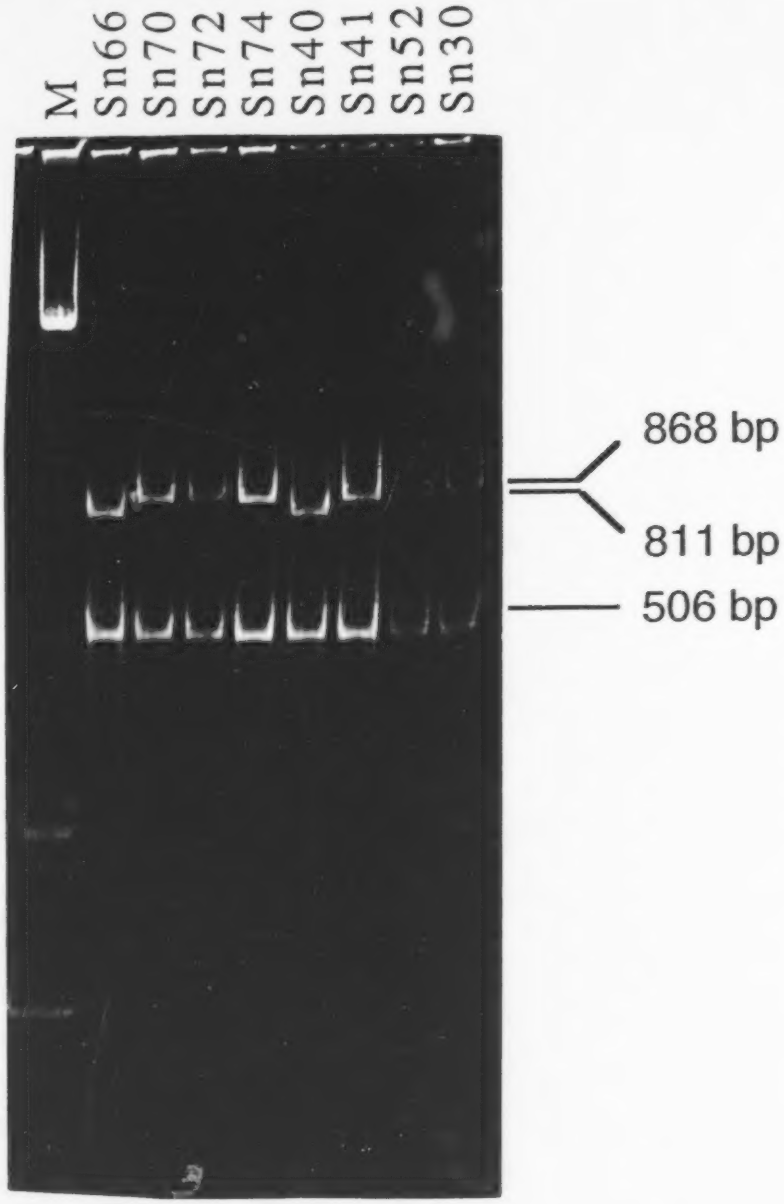
Amplifications were performed using a Perkin Elmer Cetus 480 DNA Thermocycler under the following conditions: initial denaturation at 94°C for 4 minutes, annealing at 56°C for 30 seconds, primer extension (or synthesis) for 2 minutes at 72°C, followed by forty cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, and a final cycle of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 10 minutes. Products obtained following amplification were electrophoresed on a 1% agarose gel and visualized following staining with ethidium bromide under UV light.

RFLP Analysis

Amplified PCR products were digested with eight restriction enzymes: Cfo I, Hae III, Hinf I, Dde I, Sau3A 1, Hpa II, Alu I, and Rsa I. Digests were performed in 20 μL volumes with 100-200 ng of amplified DNA, 3 U of enzyme and a 1/10 dilution of the manufacturer recommended 10x digestion buffer. Digestions were incubated overnight at 37°C and the resulting fragments were separated by gel electrophoresis on a BioRad Vertical gel apparatus using a 4% polyacrylamide 1x TBE gel buffered in 0.5x TBE at approximately 300 volts/25 mA for 2-2.5 hours. Following electrophoresis the restriction patterns were visualized by staining with ethidium bromide under UV light (see Figs. 2-2 to 2-5). Restriction patterns were photographed and their sizes were estimated by comparison with a commercially obtained 123 bp ladder (BRL).

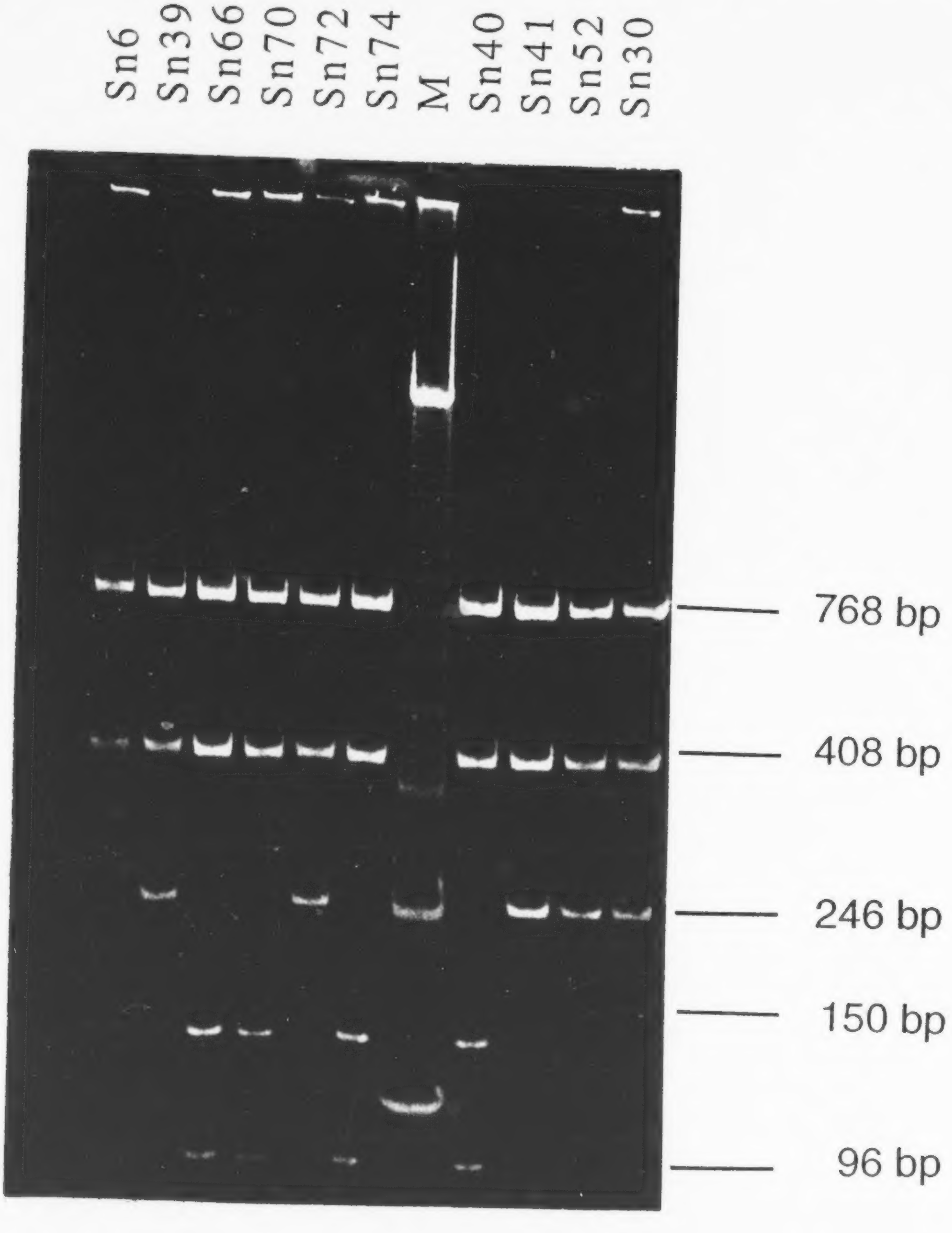
Following sizing, all variable sites were identified and the gene frequencies of haplotypes in each population were estimated. The probability that two alleles chosen at

Fig 2-2. Restriction digestion pattern obtained using the restriction enzyme Sau 3A I. The individual identification #'s of the lake trout examined are indicated above the gel; the lane marked with an M is a commercially obtained 123 bp ladder (each band increases in size by 123 bp). The sizes of the individual fragments are listed to the right of the gel image.


M
Sn66
Sn70
Sn72
Sn74
Sn40
Sn41
Sn52
Sn30
868 bp
811 bp
506 bp

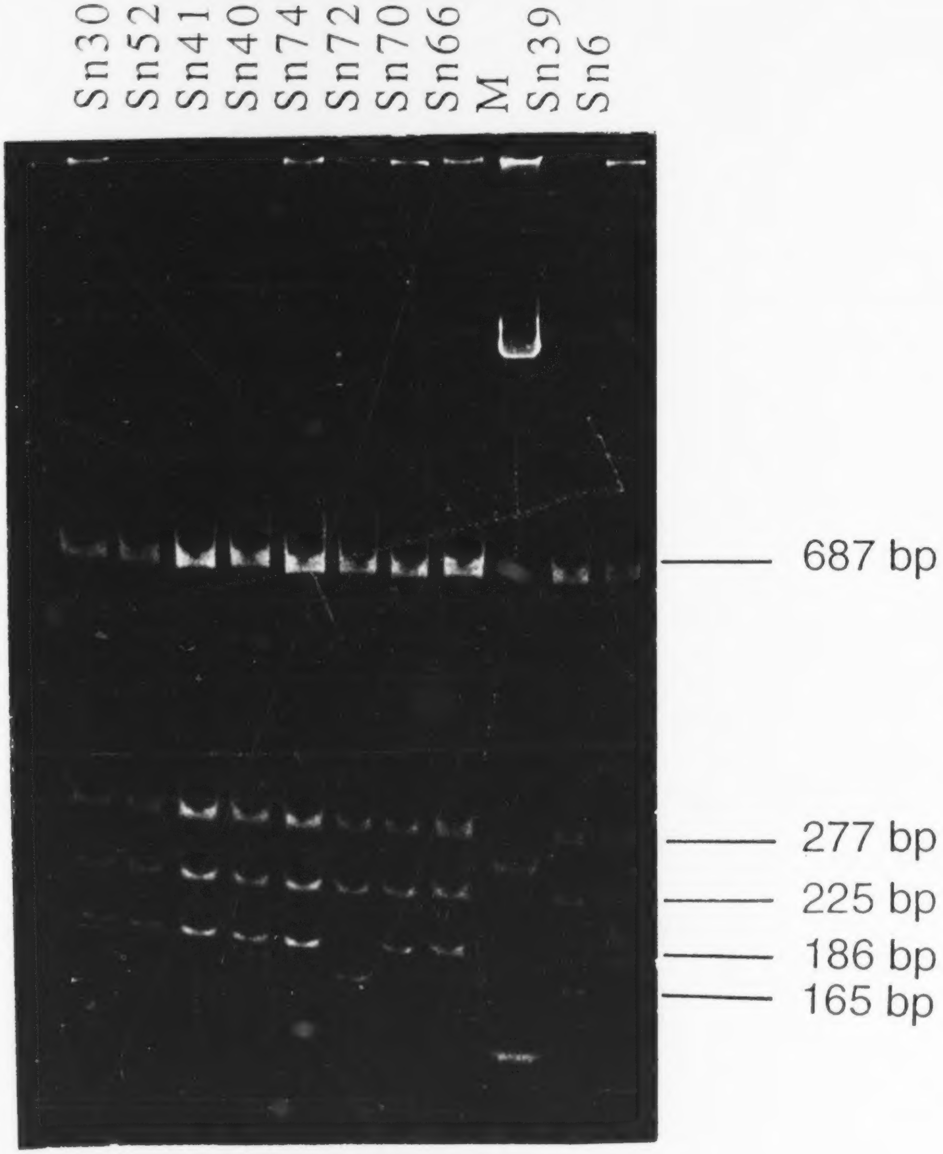
Sau 3 AI

Fig 2-3. Restriction digestion pattern obtained using the restriction enzyme Hinf I. The individual identification #'s of the lake trout examined are indicated above the gel; the lane marked with an M is a commercially obtained 123 bp ladder (each band increases in size by 123 bp). The sizes of the individual fragments are listed to the right of the gel image.


Sn6
Sn39
Sn66
Sn70
Sn72
Sn74
M
Sn40
Sn41
Sn52
Sn30
768 bp
408 bp
246 bp
150 bp
96 bp

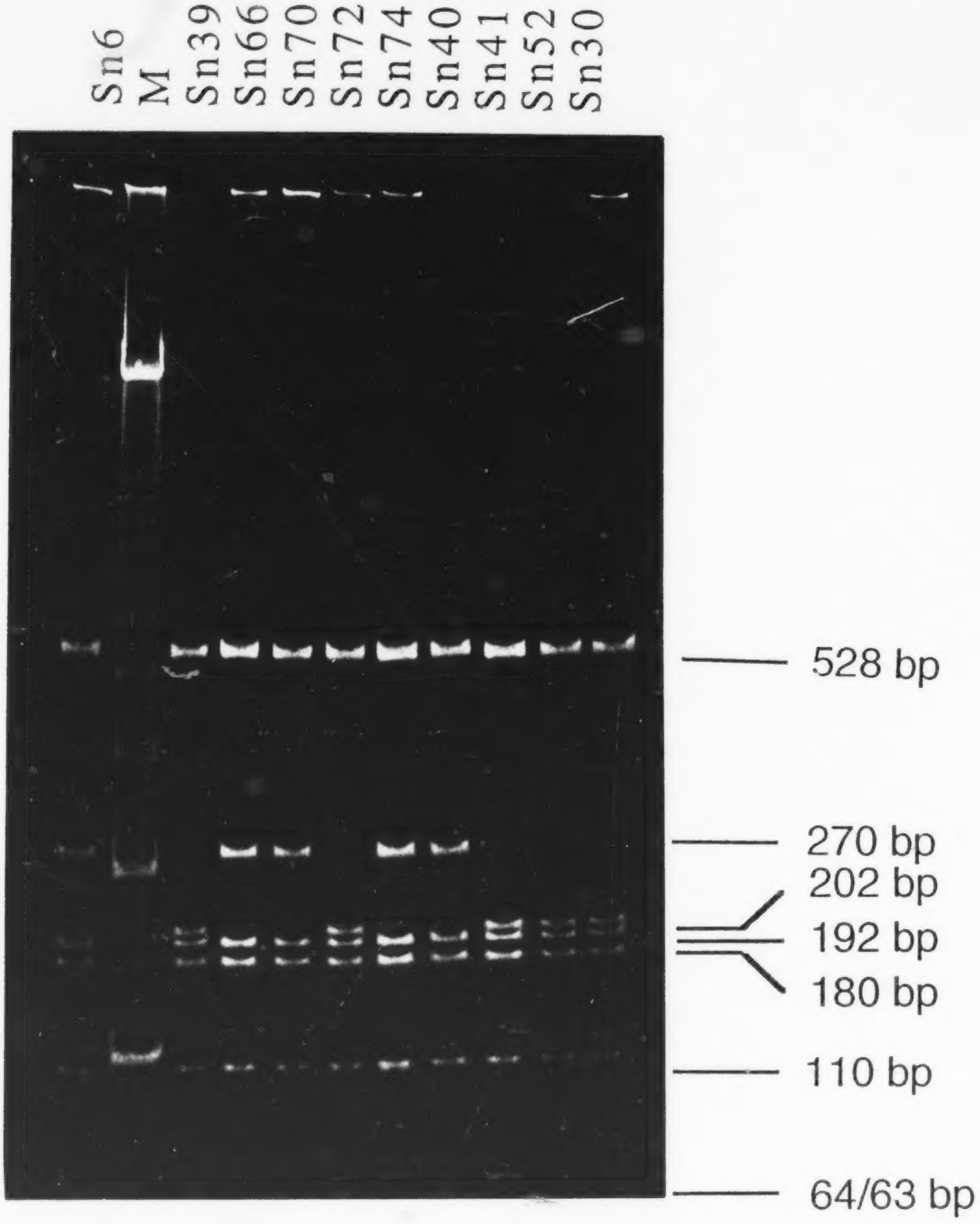
Hinf I

Fig 2-4. Restriction digestion pattern obtained using the restriction enzyme Alu I. The individual identification #'s of the lake trout examined are indicated above the gel; the lane marked with an M is a commercially obtained 123 bp ladder (each band increases in size by 123 bp). The sizes of the individual fragments are listed to the right of the gel image.


Sn30
Sn52
Sn41
Sn40
Sn74
Sn72
Sn70
Sn66
M
Sn39
Sn6
687 bp
277 bp
225 bp
186 bp
165 bp

Alu I

Fig 2-5. Restriction digestion pattern obtained using the restriction enzyme Hae III. The individual identification #'s of the lake trout examined are indicated above the gel; the lane marked with an M is a commercially obtained 123 bp ladder (each band increases in size by 123 bp). The sizes of the individual fragments are listed to the right of the gel image.


Sn6
M
Sn39
Sn66
Sn70
Sn72
Sn74
Sn40
Sn41
Sn52
Sn30
528 bp
270 bp
202 bp
192 bp
180 bp
110 bp
64/63 bp

Hae III

random from the population are different from each other, or the "gene diversity" of the examined loci were calculated for all of the populations examined (Li, 1991). As a test of significance the frequency of haplotypes between populations were compared using a Chi square test of heterogeneity (Zar, 1984). The ability to detect an individual type within a population is related to its frequency, and the size of sample taken from that population. Thus the discussion of the significance of the presence, absence, or frequency of a particular haplotype within a population must include some reference to the probability of detecting a haplotype within a population given a certain sample size. The probability of not detecting a haplotypes with theoretical frequencies of 0.05, 0.10, 0.15, 0.20, and 0.25 in a population, given varying sample sizes, were calculated (Table 2-4).

Sequencing

Amplified COIII-ATPase fragments were sequenced in 32 lake trout. Individuals were selected on the basis of their observed RFLP types and individuals of each of the RFLP types (A, B, C, or D) from each of the geographic locations were sequenced. A single brook trout, *S. fontinalis*, and a single bull trout, *S. confluentus*, were also sequenced as "out groups" for phylogenetic analysis. Sequences were obtained using the *Taq* Dye Deoxy^{TM} Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Calif.). Sequencing was performed directly on double stranded PCR product. Following initial amplification PCR products were electrophoresed on a 1% agarose gel to purify the PCR product from any unreacted primers, and secondary products. Once separated the sequence templates were excised from the agarose gel, retrieved by electroelution, and resuspended in sterile double distilled water. Sequencing using the *Taq* Dye Deoxy^{TM} Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Calif.) was accomplished using a final volume of 20 ul, with approximately 1 μg of purified PCR template, 3.2 pmol of the appropriate sequencing primer, a 9.5 ul of a Reaction Premix. Sequence products were generated using the Perkin Elmer Cetus Model 9600 Thermal Cycler. Templates were

Table 2-4. Probability of not detecting a haplotype within a population with a sample size of n individuals.

Frequency of haplotype in the population	Sample Size								
f	1	2	3	4	5	6	7	8	9
0.05	0.950	0.903	0.857	0.815	0.774	0.735	0.698	0.663	0.630
0.10	0.900	0.810	0.729	0.656	0.591	0.531	0.478	0.431	0.387
0.15	0.850	0.723	0.614	0.522	0.444	0.377	0.321	0.272	0.232
0.20	0.800	0.640	0.512	0.410	0.328	0.262	0.210	0.168	0.134
0.25	0.750	0.578	0.422	0.316	0.237	0.178	0.134	0.100	0.075

f	10	15	20	25	30	35	40	45	50
0.05	0.599	0.463	0.358	0.277	0.215	0.166	0.129	0.099	0.076
0.10	0.349	0.206	0.126	0.071	0.042	0.025	0.014	---	---
0.15	0.197	0.087	0.038	0.017	0.007	0.003	---	---	---
0.20	0.107	0.035	0.011	0.003	---	---	---	---	---
0.25	0.056	0.013	0.003	---	---	---	---	---	---

preheated to 96 ^{o}C; exposed to 25 cycles of 96 ^{o}C for 15 seconds, 50 ^{o}C for 1 second, and 60 ^{o}C for 4 minutes; samples were then held at 4 ^{o}C until they could be visualized.

Amplified reaction mixtures were loaded onto the Centri-Sep^{TM} spin columns (Princeton Separations Inc., New Jersy) to remove excess DyeDeoxy^{TM} Terminators from the completed sequence reactions. Columns were spun at 1300 *g* for 2 minutes in a Baxter Canlab Biofuge 13 with a fixed angle rotor, and collected in a 1.5 ul eppendorf tube. Reaction mixtures were dried in a Jouan RC 10-10 vacuum centrifuge and the resulting pellets were resuspended in 4 uL of a mixture of 5 uL deionized formamide/1 uL of 50 mM EDTA, pH 8.0 . Templates were heated at 90 ^{o}C for 2 minutes to denature the DNA and immediately placed on ice prior to separation on a 6 % polyacrylamide gel using an Applied Biosystems 373A DNA Sequencer. Resulting sequences were analyzed using the Applied Biosystems SeqEd^{TM} DNA Sequence Editor.

Analysis of sequences from all of the individuals were accomplished using Phylogentic Analysis Using Parsimony (PAUP) of Swofford (1991). Most parsimonious trees were calculated using the Heuristic search option, with random addition of trees for 10 replicates; a strict consensus tree was calculated from the shortest trees using the brook trout *S. fontinalis* and the bull trout *S. confluentus* for rooting as outgroups. The PAUP Bootstrap option with 100 replications was used to test the rigor of the topology of the strict consensus tree.

Results

RFLP Analysis

The ATPase-COIII regions of 174 individual lake trout were examined using the eight restriction enzymes (for a complete digest summary see Appendix I). Some fragments were too small to be consistently visualized with ethidium staining. The fragment sizes were verified by comparisons with sequence data. Of the eight restriction enzymes used, four enzymes each identified a single polymorphic site. The restriction enzymes Hae III, Alu I, Hinf I, and Sau 3A I, gave two variable restriction patterns (see Figs. 2-2 to 2-5). A 266 bp fragment in the Hae III restriction digest was cut resulting in a 202 bp fragment and 64 bp fragment that was not visible. Variation in the Alu I restriction pattern occurred when a 186 bp fragment was cut into a 165 bp fragment and a 21 bp fragment that was not visible. In the Hinf I pattern a 246 bp fragment was cut into two smaller fragments of 150 bp and 96 bp. The Sau 3A I variant exhibited either an 868 bp fragment or an 811 bp and a 57 bp fragment that was not consistently visible. No other changes in band sizes were apparent in the other bands present or other restriction patterns.

A total of four mtDNA haplotypes were identified by the RFLP digests (Tables 2-5 and 2-6), and each type differed in at least one restriction site. A comparison of the frequency distribution of the four haplotypes in the total Cold Lake population to the three donor populations shows that no RFLP haplotypes were unique to any single population, and at least one haplotype was shared between all of the examined stocks (Figure 2-6). The apparent lack of fixed variation indicates that these four haplotypes diverged genetically (at least with respect to their mtDNA) before they were physically separated and not enough time has passed for these populations to accumulate fixed variation between the populations. Although there were no unique haplotypes the frequency of haplotypes

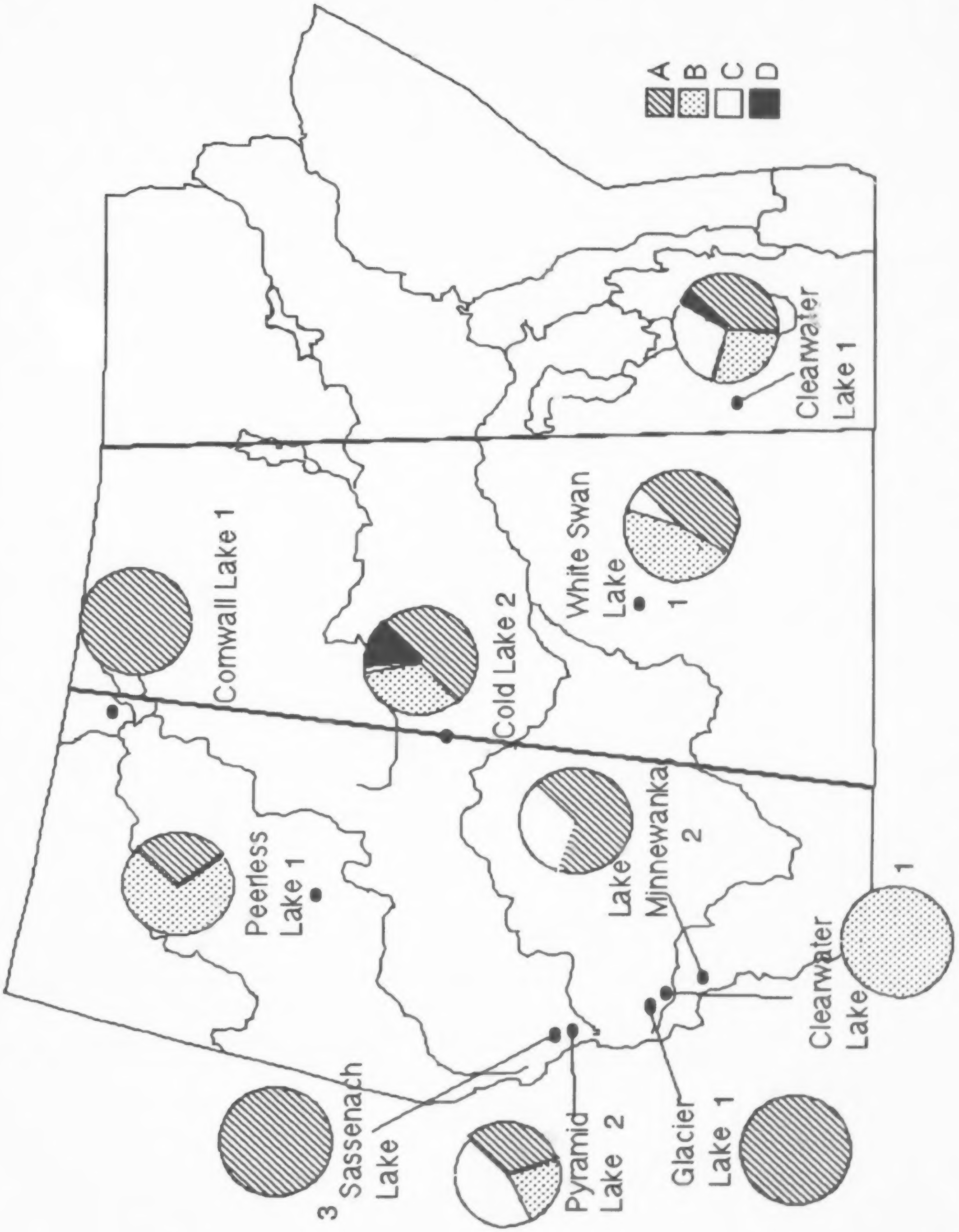
Table 2-5. Frequencies of four mitochondrial haplotypes observed among all populations examined in this study (N=the total sample size from each population). The gene-diversity or heterozygosity (h) was calculated from the observed frequencies of the mtDNA haplotypes in each population.

Location	N	Haplotype	frequency	# individuals /haplotype	gene-diversity (h)
Cornwall, AB.	8	A	1.0	8	---
Clearwater, MB.	24	A	0.38	9	0.686
		B	0.29	7	
		C	0.29	7	
		D	0.04	1	
White Swan, Sask.	26	A	0.46	12	0.574
		B	0.46	12	
		C	0.08	2	
Cold, AB. Adults	17	A	0.41	7	0.546
		B	0.53	9	
		D	0.06	1	
Sub-Adults	14	A	0.57	8	0.601
		B	0.14	2	
		C	0.07	1	
		D	0.21	3	
Juveniles	12	A	0.58	7	0.571
		B	0.25	3	
		D	0.17	2	
Clearwater BNP., AB.	4	B	1.0	4	---
Pyramid, JNP., AB.	9	A	0.33	3	0.643
		B	0.22	2	
		C	0.45	4	
Sassenach, JNP., AB.	15	A	1.0	15	---
Glacier, BNP., AB.	15	A	1.0	15	---
Minnewanka, BNP., AB.	19	A	0.68	13	0.438
		C	0.32	6	
Peerless, AB.	11	A	0.27	3	0.407
		B	0.72	8	

Table 2-6. Frequencies of four mitochondrial haplotypes observed in the Cold Lake population and the total gene-diversity or heterozygosity (h) from the combined frequencies of each subpopulation (N=the total sample size from each population). The sample size, frequencies of each subpopulation were taken from Table 2-5.

Location	N	Haplotype	frequency	# individuals /haplotype	gene-diversity (h)
Cold Lake	43	A	0.51	22	0.61
		B	0.33	14	
		C	0.02	1	
		D	0.14	6	

Figure 2-6. The % composition and distribution of the four haplotypes identified by restriction analysis in all ten lake trout populations examined. Percentages and sample sizes from each population were taken from table 5. Lakes with different stocking histories were labeled as follows: 1 = endemic population; 2 = endemic population that have been stocked; 3 = stocked population.

▨ A
▒ B
☐ C
■ D
Clearwater Lake 1
White Swan Lake 1
Comwall Lake 1
Cold Lake 2
Peerless Lake 1
Lake Minnewanka 2
Clearwater Lake 1
3 Sassenach Lake
Pyramid Lake 2
Glacier Lake 1

between Cold Lake and the donor lakes was significantly different (Chi^2=27.029; d.f.=9; p>0.005). The overall composition of haplotypes between generations in the Cold Lake stock was not significantly different (Chi^2=7.855; d.f.=6, p<0.10) and were grouped as one homogeneous population for further comparisons (Table 2-6).

Of the four haplotypes observed in Cold Lake and the three donor lakes: Cornwall Lake, Alberta; Clearwater Lake, Manitoba, and; White Swan Lake, Saskachewan, the most common was A. It was present in all of the populations (see Table 2-5). The lowest frequency of A was observed in the Clearwater Lake, Manitoba (0.38). The highest frequency was observed in the Cornwall Lake population (1.0) Only eight fish from Cornwall Lake were examined, and the failure to detect any other haplotypes in this lake may be due to the small sample size from this lake. The B haplotype was also present in two of the donor stocks and all of the Cold Lake subpopulations. The co-occurrence of two distinct mitochondrial lineages in several populations suggest that these populations can not be distinguished phylogeneticaly. Clearwater Lake, Manitoba, exhibited the greatest degree of gene diversity (0.686), and shared all four haplotypes with the Cold Lake subadult subpopulation. The C type was present in low to moderate frequencies in two of the donor lakes, 0.08 to 0.29, but was present in low frequencies in the Cold Lake population, and was only found in the sub-adult population. This would suggest that this type may have been introduced from one of the donor lakes. The D type had the most limited distribution. It was present in only two lakes, and exhibited the lowest frequencies 0.04 for Clearwater Lake, MB, and 0.14 from the total sample from Cold Lake. This suggests that the D haplotype was introduced from the Clearwater stock. Seventeen individuals were sampled from the Cold Lake adult population and the probability of not detecting this haplotype in the adult population, given its observed frequency in the subadult population (0.07), was 29%.

The same mtDNA haplotypes observed in the Cold Lake and donor lakes, with the exception of the D type, were also observed in the six other Alberta lake populations. No new haplotypes were identified (Table 2-5 and Figure 2-6). A chi square test of heterogeneity did indicate that significant differences do exist in the frequencies of the different haplotypes between all of the populations examined (Chi^2=101.8967; d.f.=27; p>0.001). The A type was present in all of the populations examined except for Clearwater Lake, B.N.P., but the failure to detect the A type in Clearwater Lake, B.N.P., is likely related to the small sample size taken from this lake (probability of not detecting a haplotype with a frequency of 0.25 in the population was 0.422). The C type was present in Pyramid Lake, J.N.P., and Lake Minnewanka, B.N.P., in relatively high frequencies. The D haplotype did not occur in any other Alberta lake trout populations. Comparisons of the gene diversity within all of the populations examined indicated that Cornwall lake, MB, has the highest level of gene diversity (0.686) followed by Pyramid Lake in Jasper National Park (0.643), AB, and Cold Lake (0.61), AB. The lowest levels of gene diversity were observed in Peerless Lake (0.407), AB, and Lake Minnewanka, B.N.P., (0.438).

Sequence Analysis

Based on the observed RFLP haplotypes, individuals of each type (A, B, C, D) from each of the populations examined were sequenced to examine the phylogenetic relationships among the four RFLP types. Not all of the observed RFLP haplotypes from each of the populations were sequenced. The single D type in the Clearwater Lake, MB., and the C type from White Swan Lake, SASK., were omitted from the sequence analysis. The complete structure and sequence composition of the sequences obtained in this study are described in Chapter 1 of this thesis. All of the sequence variations observed in the 32 individual *S. namaycush* sequences were single base pair substitutions with 18 variable nucleotide sites and a total of 17 different composite haplotypes were identified (Table 2-7). Of the 18 variable nucleotide sites identified, 10 were informative, occurring in two or

Table 2-7. Character states and position of variable nucleotide positions obtained from the ATPase-6/CoIII sequence of 32 individual *Salvelinus namaycush*, a single *Salvelinus confluentus*, and a single *Salvelinus fontinalis*. Individuals were taken from 10 different locations and are grouped according to their respective haplotypes.

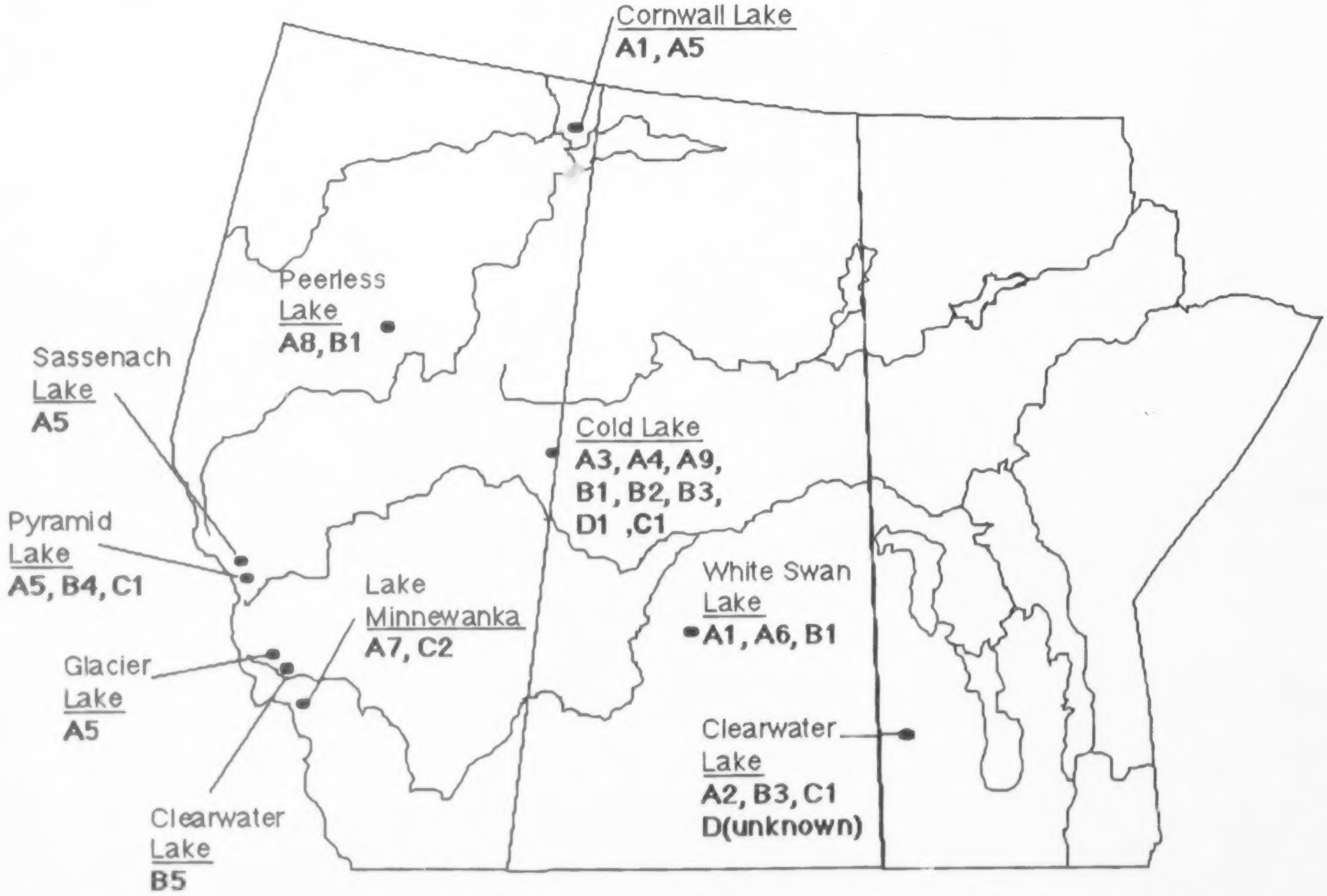
ID	Type	Site Number																	
		261	342	354	454	532	591	618	645	919	957	995	1133	1223	1272	1280	1334	1353	1386
Sn 97	B1	G	C	A	C	G	T	A	C	G	T	C	G	G	G	G	A	T	G
Sn 52	B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sn 23	B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sn 151	B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sn 45	B2	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sn 30	B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
Sn 184	B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
Sn 15	B3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-
P 8	B4	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CB 2	B5	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-
Sn 53	D1	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
Sn 39	D1	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
Sn 36	D1	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
Sn 78	C1	A	-	-	-	-	-	-	A	-	-	-	A	A	-	A	-	-	-
Sn 74	C1	A	-	-	-	-	-	-	A	-	-	-	A	A	-	A	-	-	-
Sn 70	C1	A	-	-	-	-	-	-	A	-	-	-	A	A	-	A	-	-	-
P 6	C1	A	-	-	-	-	-	-	A	-	-	-	A	A	-	A	-	-	-
Sn 163	C2	A	-	-	-	-	-	-	A	T	-	-	A	A	-	A	-	-	-
Sn 3	A1	A	G	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
Sn 94	A1	A	G	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
Sn 75	A2	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	A
Sn 71	A3	A	-	-	-	A	-	G	A	-	C	-	-	A	-	A	-	-	-
Sn 66	A4	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	T	-	-
Sn 132	A5	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
S 5	A5	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
Sn 4	A5	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
P 7	A5	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
Sn 27	A6	A	-	-	-	-	C	G	A	-	-	-	-	A	-	A	-	-	-
Sn 165	A7	A	-	-	-	-	-	G	A	-	-	-	-	A	-	A	-	-	-
Sn 155	A8	A	-	-	-	-	-	G	A	-	-	-	A	A	-	A	-	-	-
Sn 161	A8	A	-	-	-	-	-	G	A	-	-	-	A	A	-	A	-	-	-
Sn 49	A9	A	-	-	-	A	-	G	A	-	-	-	-	A	-	A	-	-	-
Sc 1	Bull Trout	-	-	-	-	-	-	G	-	-	-	A	-	A	-	A	-	-	-
Sf 72	Brook Trout	A	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-

more individuals sequenced, and the rest consisted of single unique base pair changes. The four polymorphic restriction sites identified by RFLP analysis were identified by sequence analysis: the Sau 3A I site is the result of a single A-G base change at bp 618; the Alu I site is the result of a single C-T change at bp 995; the Hae III site is the result of a single G-A change at bp 1223; the Hinf I site was the result of a single G-A change at bp 1280. Six more phylogeneticaly informative sites were identified by sequence analysis, none of which resulted in any further restriction sites.

The unique haplotypes identified by sequencing are first labeled with the restriction digest designation (i.e. belonging to the A, B, C, or D groups) followed by a number denoting the haplotype identified by sequencing. The sequence analysis revealed a larger number of unique haplotypes that were not detected with the RFLP analysis. The A group of mitochondrial haplotypes was the most diverse with 9 haplotypes; five types were identified in the B group; two types in the C group; and a single type from the D group. A total of 12 haplotypes occurred in only one location (Figure 2-7). The remaining 5 types occurred in at least two populations. The A5 type was the most common type, occurring in 50% of the populations examined. The occurrence of multiple types in different geographic locations concurs with the same observation from the restriction analysis. The number of individuals sequenced ranged from a single individual in two of the populations examined, to 10 individuals from Cold Lake. The small sample sizes limit the conclusions regarding the distribution of the sequence types and their frequency in the populations examined, but suggests the occurrence of rarer unique haplotypes in these populations.

Phylogenetic analysis of the sequence data resulted in six most parsimonious trees of differing topologies (Figure 2-8). The 32 sequence haplotypes separated into two evolutionary divisions; the B and D types and the A and C types. Bootstrap analysis revealed that the ambiguity in the branching order of individual lake trout was restricted primarily to the A and C types (Figure 2-9). The A group of haplotypes separated

Figure 2-7. Composition and distribution of haplotypes identified by sequence analysis in all ten lake trout populations examined. The numbers of individuals sequenced varied from each population: White Swan, Sask, n=4; Clearwater, MB, n=5; Cornwall, AB, n=2, Cold, AB, n=10; Peerless, AB, n=3; Pyramid (J.N.P.), AB, n=3; Sassenach (J.N.P.), AB, n=1; Clearwater (B.N.P.), AB, n=1; Glacier (B.N.P.), AB, n=1; Minnewanka (B.N.P.), AB, n=2.


Cornwall Lake
A1, A5
Peerless Lake
A8, B1
Sassenach Lake
A5
Cold Lake
A3, A4, A9, B1, B2, B3, D1 ,C1
Pyramid Lake
A5, B4, C1
Lake Minnewanka
A7, C2
White Swan Lake
A1, A6, B1
Glacier Lake
A5
Clearwater Lake
A2, B3, C1 D(unknown)
Clearwater Lake
B5

phylogeneticaly into three major divisions. Two closely related types the A3 and A9 haplotypes branch off from all the other A types but occur only in the Cold Lake population. The A1 haplotype also separates out from all other A types but is shared between both White Swan Lake and Cornwall Lake. Sequence analysis of two individuals from the Peerless Lake population revealed a unique A8 haplotype that shared a single informative site with the C group of haplotypes, suggesting a recent division of the A8 type from the C group of fish. The C group of fishes consist of two haplotypes that are closely related to the A group. The phylogenetic relationships between the rest of the A haplotypes is unclear as the remaining A types form a polytomy. The B haplotypes separated strongly into two groups consisting of the B3 haplotype phylogeneticaly separate from the remaining four B haplotypes. All of the D types consisted of a single haplotype derived from the B haplotypes.

Discussion

Restriction fragment analysis identified four mitochondrial haplotypes. Further sequence analysis of a protein coding region spanning both the ATPase-6/COIII genes revealed a total of 17 mitochondrial haplotypes. Haplotypes unique to a particular geographic location co-occured with more commonly occurring types. The occurrence of several lake trout mtDNA lineages in more than one geographic location and the co-occurence of several haplotypes unique to single locations suggest two possibilities: 1) a recent geographic separation of these populations and a limited degree of genetic differentiation between populations; 2) extensive human transfers and introductions of lake trout to geographically isolated populations. In the first instance, not enough time has elapsed to allow for fixed variation to be distinguished between these populations by mitochondrial DNA. Mounting evidence suggests a slow rate of genetic change in the mitochondrial genomes of some fishes (Rand 1993); particularly in the salmonid fishes (Guiffra *et al.*, 1994; Bernatchez and Danzman, 1993; and Chapter 1 of this thesis). Thus

Figure 2-8. A 50% majority consensus tree of six most parsimonious trees calculated from sequences obtained for the ATPase-6/COIII region of the mitochondrial DNA of 32 individual *S. namaycush*, a single *S. confluentus*, and a single *S. fontinalis*. Trees were generated using the Heuristic search function of PAUP (Swofford 1992), with random addition of trees (10 replicates) and the multipars option in effect. The numbers on the tree branches represent the occurrence of a particular branch in the six trees. Frequencies for branches below 50% were omitted and were considered interchangeable at those branch points.

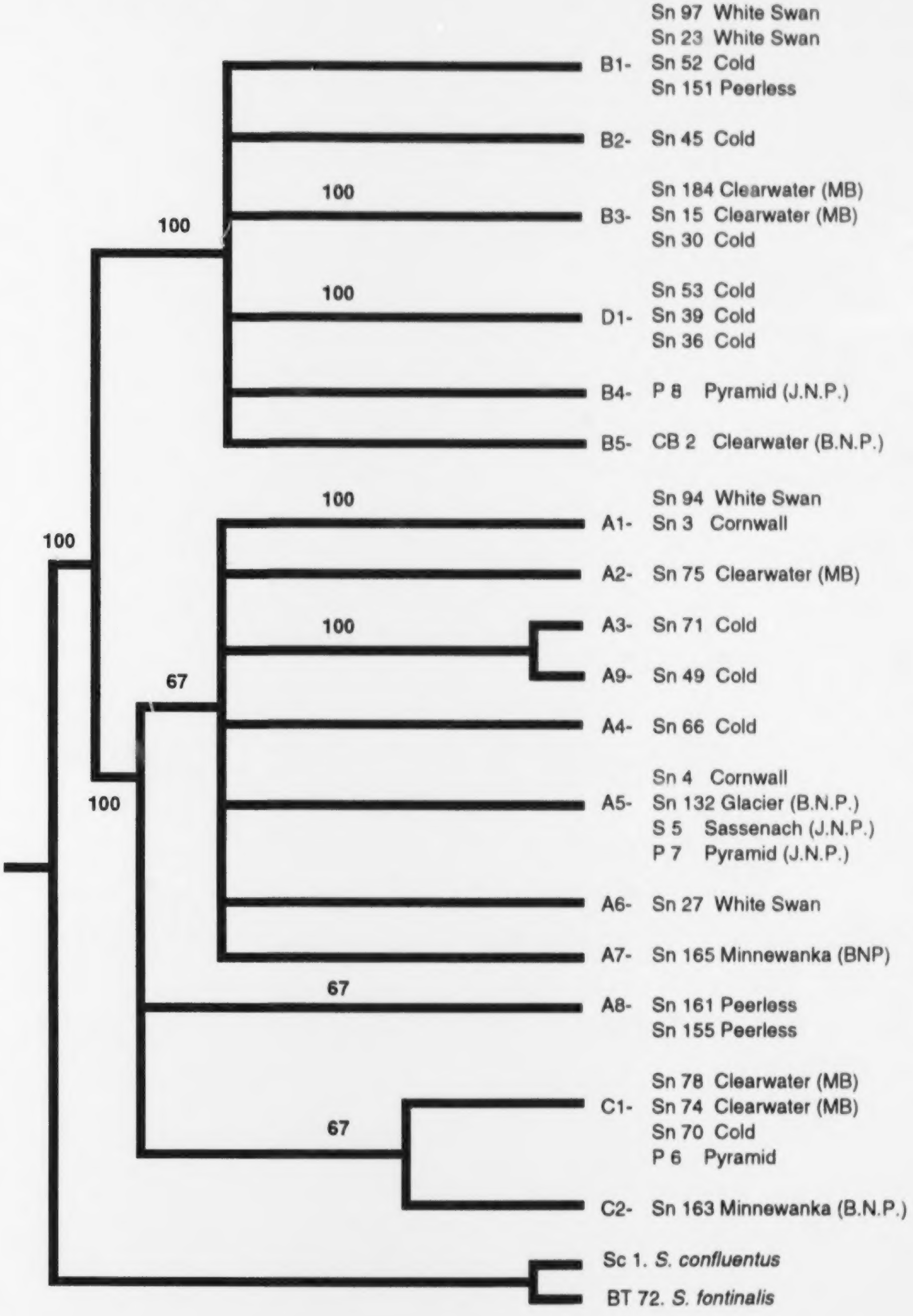
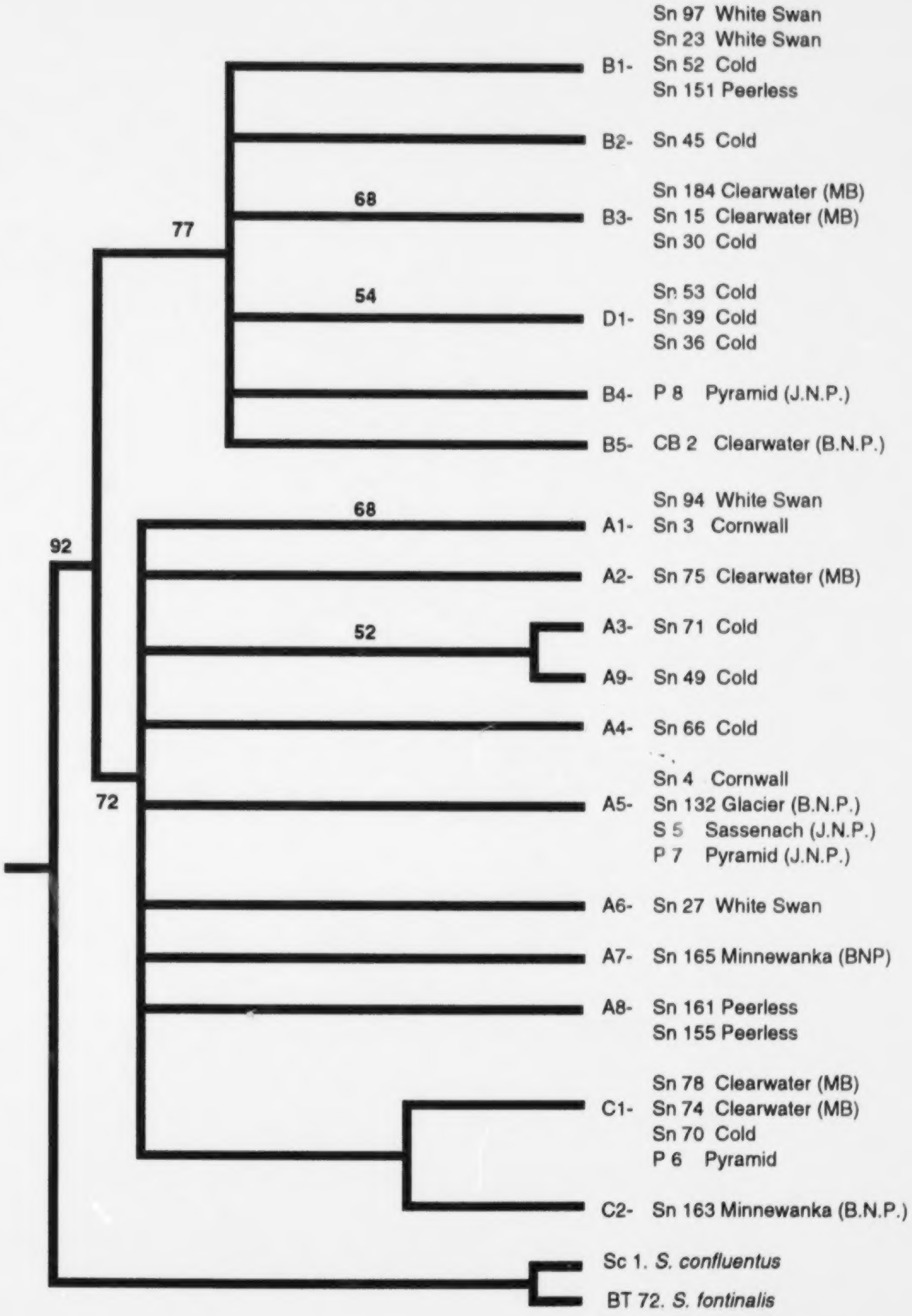

100
100
100
100
100
67
100
100
67
67
B1- Sn 97 White Swan
Sn 23 White Swan
Sn 52 Cold
Sn 151 Peerless
B2- Sn 45 Cold
B3- Sn 184 Clearwater (MB)
Sn 15 Clearwater (MB)
Sn 30 Cold
D1- Sn 53 Cold
Sn 39 Cold
Sn 36 Cold
B4- P 8 Pyramid (J.N.P.)
B5- CB 2 Clearwater (B.N.P.)
A1- Sn 94 White Swan
Sn 3 Cornwall
A2- Sn 75 Clearwater (MB)
A3- Sn 71 Cold
A9- Sn 49 Cold
A4- Sn 66 Cold
A5- Sn 4 Cornwall
Sn 132 Glacier (B.N.P.)
S 5 Sassenach (J.N.P.)
P 7 Pyramid (J.N.P.)
A6- Sn 27 White Swan
A7- Sn 165 Minnewanka (BNP)
A8- Sn 161 Peerless
Sn 155 Peerless
C1- Sn 78 Clearwater (MB)
Sn 74 Clearwater (MB)
Sn 70 Cold
P 6 Pyramid
C2- Sn 163 Minnewanka (B.N.P.)
Sc 1. *S. confluentus*
BT 72. *S. fontinalis*

Figure 2-9. Bootstrap of sequence data obtained for the ATPase-6/COIII region of the mitochondrial DNA of 32 individual *S. namaycush*, a single *S. confluentus*, and a single *S. fontinalis*. Bootstrap analysis was performed using PAUP's heuristic search function with 100 replicates and the Multipars option and random tree addition (10 replicates). The numbers on the branches represent consistency index of each branch above obtained from the 100 bootstrap replicates. Values below 50% are omitted from the tree and were considered interchangeable at those branch points.



92
77
68
54
68
52
72
B1- Sn 97 White Swan
Sn 23 White Swan
Sn 52 Cold
Sn 151 Peerless
B2- Sn 45 Cold
B3- Sn 184 Clearwater (MB)
Sn 15 Clearwater (MB)
Sn 30 Cold
D1- Sn 53 Cold
Sn 39 Cold
Sn 36 Cold
B4- P 8 Pyramid (J.N.P.)
B5- CB 2 Clearwater (B.N.P.)
A1- Sn 94 White Swan
Sn 3 Cornwall
A2- Sn 75 Clearwater (MB)
A3- Sn 71 Cold
A9- Sn 49 Cold
A4- Sn 66 Cold
A5- Sn 4 Cornwall
Sn 132 Glacier (B.N.P.)
S 5 Sassenach (J.N.P.)
P 7 Pyramid (J.N.P.)
A6- Sn 27 White Swan
A7- Sn 165 Minnewanka (BNP)
A8- Sn 161 Peerless
Sn 155 Peerless
C1- Sn 78 Clearwater (MB)
Sn 74 Clearwater (MB)
Sn 70 Cold
P 6 Pyramid
C2- Sn 163 Minnewanka (B.N.P.)
Sc 1. *S. confluentus*
BT 72. *S. fontinalis*

the genetic differentiation of mitochondrial DNA in lake trout and the fixation of genetic differences within populations since the most recent glacial period (20,000 to 13,000 years ago) may not have occurred.

Human influence may be a possible explanation for the shared haplotypes occurring in several locations, given the past history of stocking practices and the possibility for the introgression of types from adjacent populations. For example, the A5 haplotype was the most commonly distributed haplotype occurring in at least two populations that have received heavy supplemental stocking, and at least one population that was introduced (Figure 2-8). This suggests an "introduced" origin for this haplotype. Unfortunately the origin of the A5 haplotype can not be fully resolved with the limited sample size and number of populations in this study. The A1, B1, B3, and C1 haplotypes also occur in more than one location and may represent introduction events during the history of the resident populations of fish. Some of the highest levels of mtDNA diversity were observed in both the Cold Lake population and the Pyramid Lake population (RFLP analysis sample size = 57 and 9 respectively; numbers of individuals sequenced = 10, 3 respectively). Although limited by the sample size from each population, the higher mtDNA diversity may be related to the frequent fish introductions to these populations.

The Cold Lake and Clearwater Lake, MB, populations shared the B3 and C1 haplotypes. The B1 type in Cold Lake was also detected in White Swan Lake. The D type identified by the RFLP analysis was detected only in the Cold Lake and Clearwater Lake populations. These results would indicate that the introductions to Cold Lake, with fish from Clearwater Lake, MB, and possibly White Swan Lake, have been successful. This is also supported by the observation of an increase in the recruitment of lake trout in Cold Lake, particularly the recruitment of fish with similar morphological characteristics to the more southerly populations of Clearwater Lake, MB, and White Swan Lake (G. Sterling personal communication). The population of lake trout in Cold Lake has experienced a decline in the recruitment of fish since about 1965 as a result of overfishing and reproductive failure due to significant residues of DDT in the fish (Paetz and Zelt, 1974; Roberts, 1975; Miller, 1965). The recovery of the lake trout population of Cold Lake in the 70's failed to meet the fisheries expectations, but the recent increase in the recruitment of fish and the findings of this study suggest the introduction of fish from at least two of the donor lakes has been successful.

Summary

Although the patterns of distribution of the mtDNA haplotypes identified in this study suggest certain possibilities with regards to the origins and transfer of fish between the respective lakes, analysis of the mtDNA of lake trout failed to unambiguously assign individual fish to a particular local population. The conclusions in this study are limited by both the numbers of fish examined in each population and the numbers of populations examined over a limited geographic region. These limitations aside, the observation of the co-occurence of identical haplotypes in distant populations, and the presence of unique types between local populations suggest a limited degree of genetic divergence between populations. Examinations of lake trout populations across their entire natural range in Canada, and artificially propagated populations in countries such as the continental United States, New Zealand, Sweden, and South America, would provide valuable insight into the levels of genetic variation observed in natural and introduced populations of lake trout.

The utility of mtDNA in the genetic characterization and monitoring of the changes in the genetic resources of species with the low levels of genetic divergence, such as those observed in the lake trout populations in this study require a more intensive sampling of the genomes to identify molecular markers useful for stock identification. Recently developed techniques for examining genetic variation in nuclear DNA, such as DNA fingerprinting, single-locus RFLP analysis, and analysis using microsatellite probes provide alternative techniques for the comparative analysis and quantification of genetic variation in stocks and populations of lake trout. A combined analysis of both mtDNA and highly variable nuclear loci should provide adequate resolution to resolve inquiries into the stock and population structure of slowly diverging fish species.

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Chapter 3

A Preliminary Examination of the mtDNA Diversity Within the Athabasca Rainbow Trout, *Oncorhynchus mykiss*.

Introduction

The purpose behind phylogenetic inference is to trace the "most-likely" evolutionary states that give rise to extant taxa (Behnke, 1992). In the case of organisms such as members of the Salmonidae, evolutionary relationships are often obscured at both the generic and species levels, by major differences in the life histories of individual species, and the possibility of interbreeding between species (Behnke, 1992; Nelson and Paetz, 1992; Wilson and Hebert, 1994). Although rainbow trout are currently classified as members of the genus *Oncorhynchus* (Robbins *et al.*, 1991), rainbow, cutthroat, and golden trout were considered members of the genus *Salmo* into the late 1980s (Bailey and Robbins, 1988). The recent reclassification of the genus *Oncorhynchus* has not fully resolved questions regarding the evolutionary relationships at the generic and specific levels of the western trouts and is accepted with some trepidation (Stearly and Smith, 1993; Nelson and Paetz, 1992).

Behnke (1992) recognized several major evolutionary groups within the red band or rainbow trout complex: red band trout of the Columbia River basin, both east of the Cascade Mountains and in the upper Fraser River basin (classified as *O. mykiss gairdneri*); redband trout of the Sacramento River basin, which is divided into two Kern River drainage subspecies, *O. m. aguabonita* and *O. m. gilberti*; the McCloud River subspecies (provisionally denoted as *O. m. stonei*); the coastal rainbow trout *O. m. irideus* and *O. m. mykiss* of east Asia. In addition to the major evolutionary lines listed here, several populations of redband trout can not be "consistently" assigned to the above groups, one such group is a population of trout native to the headwaters of the Athabasca River drainage.

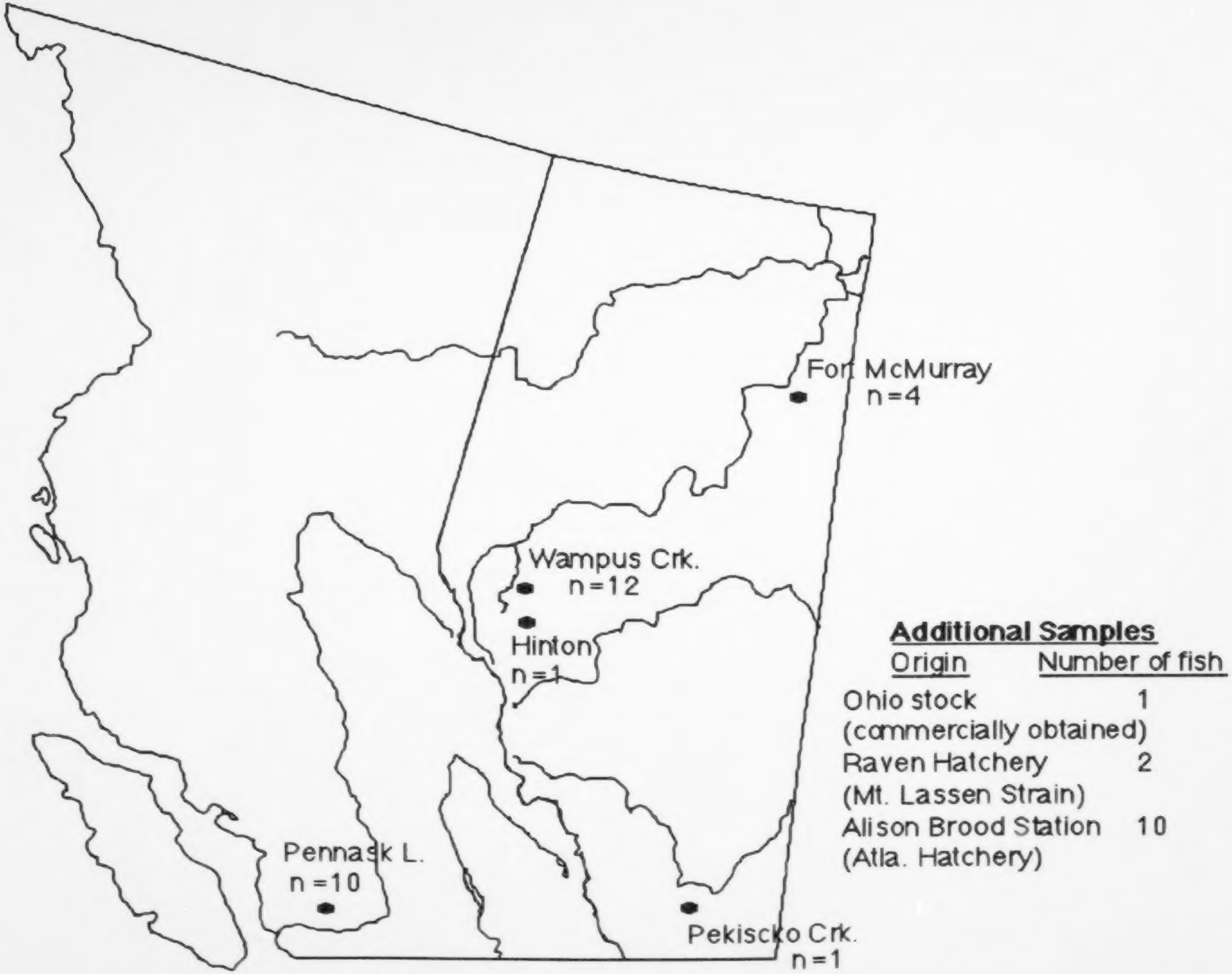
Wampus Creek, a tributary of the McLeod River which is part of the Athabasca River drainage contains a population of rainbow trout that is believed to be a pure strain of the Athabasca Rainbow Trout. This strain of rainbow trout exhibits unique characteristics that identify it as a distinct strain of rainbow trout. Trout from the Athabasca headwaters have similar coloration and pyloric caeca counts to other inland redband populations, but similar scale counts to coastal populations (Mayhood, 1992; Behnke, 1979; Bajkov, 1927). Moreover, the Athabasca trout differ at several allozyme loci from both coastal rainbow populations and inland redband trout populations (Leon Carl *et al.*, Ontario Ministry of Natural Resources. Research Section, Fisheries Policy Branch in press; Mayhood, 1992). There are significant life history differences between the Athabasca stock of rainbow trout and other rainbow trout populations (Nelson and Paetz, 1990). Spawning occurs much later than introduced southerly rainbow trout and the Athabasca stock is the slowest growing rainbow trout in the world (Nelson and Paetz, 1992; Sterling, 1990). Morphological, meristic, and allozyme electrophoresis data suggest that the Athabasca Rainbow Trout diverged from any form of redband or coastal rainbow trout prior to the last glacial period, ~64,000 years ago. This stock of rainbow trout could represent a separate evolutionary group from other stocks of rainbow trout. The unique life history characteristics such as delayed spawning times and slow growth rates suggest the possibility of little or no hybridization between this unique population and other introduced stocks of rainbow trout.

Reproductive isolation of the Athabasca trout prior to the last glacial period suggests survival in a north-western glacial refugium. This is contrary to current hypotheses regarding the post glacial colonization of the Athabasca River from headwaters of the Fraser drainage. Geographical patterns and extant distributions of coastal and inland rainbow trout suggest that coastal rainbow trout did not survived the last glacial period in a northern refugium (Behnke, 1992; Lindsey and McPhail, 1986). A hypothesis for the existence of glacial refugium in western Canada gains support from the occurrence of several unique species and subspecies in the Athabasca drainage. The occurrence of organisms such as the Jasper longnose sucker (*Catastomus catastomus lacustris*), the Banff longnose dace (*Rhinichthys cataractae smithi*), the Athabasca pearl dace (*Margariscus margarita* ssp.), two endemic species of snails (*Physa johnsoni* and *Physa jennessi* *athearni*), and the only North American *Acroloxidae* species of freshwater limpet (*Acroloxus coloradensis*), support the possibility of a glacial refugium (see Schindler *et al.* for a review of species unique to waters of the Canadian mountain National Parks). Thus the possibility of a glacial free corridor in western Alberta cannot be ruled out (for further discussion see Mayhood, 1992; Crossman and McAllister, 1986).

Morphological detection of hybridization within salmonid species such as the rainbow trout is difficult because hybrids often fail to exhibit morphological intermediacy between parent taxa (Leary *et al.*, 1985). Direct examination of the DNA facilitates the detection of hybridization events when little or no morphological differences exist between hybrids and parental stocks. In species that hybridize as readily as rainbow trout mtDNA may act as a record of past hybridization events. The maternal mode of inheritance and the rates of divergence of mitochondrial DNA (mtDNA) may facilitates the detection of hybridization events. Following hybridization and subsequent back crossing with the paternal strain of fish, genetically distinct types become rapidly fixed for different mtDNA haplotypes. Gene frequencies of variable nuclear loci gradually degrade under the same circumstances showing no evidence of past isolation (Billington and Hebert, 1991; Avise *et al.*, 1987). Thus, descendants from past hybridization events exhibiting both morphological and nuclear genetic characteristics of one parental strain may contain the mtDNA haplotype of the other parent. Back crossing with the maternal strain of fish could result in the opposite effect, swamping of the mtDNA genome of the hybrid, thereby obscuring any past hybridization events. In such a case the only means of detecting the past hybridization event is to examine the nuclear DNA. This preliminary survey deals with the level of mtDNA divergence that may exist between populations of rainbow trout.

Due to the possibility of introgressive hybridization with introduced stocks of rainbow trout, genetic analysis of individuals from the Wampus Creek population of Athabasca Rainbow Trout were undertaken to identify differences in the mtDNA that would support the unique status of the Athabasca stock, and determine the degree of hybridization

Figure 3-1. Source locations and sample sizes of the rainbow trout used in this study. Additional hatchery samples and fish obtained from areas not indicated on the map are listed adjacent to the map.


Fort McMurray
n=4
Wampus Crk.
n=12
Hinton
n=1
Pennask L.
n=10
Pekiscko Crk.
n=1

Additional Samples

Origin	Number of fish
Ohio stock (commercially obtained)	1
Raven Hatchery (Mt. Lassen Strain)	2
Alison Brood Station (Atla. Hatchery)	10

between this unique stock of fish and introduced stocks of rainbow trout. In this study two regions within the mtDNA of individual rainbow trout were examined by digestion with four base restriction enzymes to examine the amount of genetic differentiation that exists between the Athabasca strain of rainbow trout to other rainbow trout in Alberta.

Materials and Methods

A total of 40 individuals from several source locations were examined (Figure 3-1); eleven individuals from the Wampus Creek population of rainbow trout; ten individuals from the Pennask Lake Brood Stock; two individuals from the Raven Hatchery (Mt. Lassen Strain), ten individuals from the Alberta Hatchery at the Alison Creek Brood Station, four individuals from the Fort McMurray region, a single fish from the Hinton area, a single fish from an Ohio stock of rainbow trout, and a single individual from Pekisco Creek in southern Alberta. Fish from wild populations and hatchery stocks were collected using a combination of creel survey, egg take and gill netting. Athabasca Rainbow Trout from the Wampus Creek population, rainbow trout from Alberta wild populations, and the Alison Brood Stock were supplied by George Sterling of Alberta Fish and Wildlife. Rainbow trout from Pennask Lake, British Columbia, were supplied by the Fish and Wildlife Research Group at the University of British Columbia.

Following collection, fish were delivered to the University of Alberta DNA Repository. Samples of 0.5 grams of hypaxial muscle were frozen in liquid nitrogen, homogenized by hand with a mortar and pestle, and resuspended in 1 ml of 0.15 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM disodium EDTA (ACK) lysis buffer at 4 ^{O}C. Total genomic DNA was then extracted using an Applied Biosystems Genepure 41 Automated DNA Extractor . Total genomic DNA was resuspended in a 1x Tris EDTA buffer and stored at -20 ^{O}C for subsequent genetic analysis.

Approximately 2400 base pairs (bp), the non-coding displacement loop or control region, along with a region of DNA spanning both the ATPase subunit 6 (ATPase 6) and the cytochrome oxidase subunit 3 (COIII) genes (see Chapter 1), were examined for genetic differences that would identify individuals from different stocks of rainbow trout. The primers used in this portion of the study were identical to those described in the first chapter of this thesis. Two primers spanning the highly conserved tRNA genes flanking the control region, CST 144 and CST 145 were constructed from published rainbow trout sequence (Digby *et al.*, 1992; Shedlock *et al.*, 1992). The second region of mtDNA examined was the 1433 bp region spanning portions of the ATPase 6 and COIII genes. Sequence data provided by Thomas and Beckenbach (1989), from 6 salmonid species, were used to construct primers CST 55 and CST 54 (see Chapter 1 for complete primer sequences).

Target regions of DNA were amplified by PCR in 100 µL reaction volumes for both the control region and ATPase-COIII genes. Reaction mixtures were adjusted to optimize yields of amplified PCR product and contained 200 µM of each primer, 0.1 mM of each deoxynucleotide dATP, dTTP, dCTP and dGTP, 1x *Thermas aquaticus* (*Taq*) magnesium free polymerase buffer (Promega, Madison WI), 2.5 µM magnesium chloride, 2.5 U of *Taq* polymeraze (Promega, Madison, WI), enough sterile double distilled water to make a final volume of 100 µL respectively, and 10-1000 ng of total genomic DNA. Reaction mixes were overlaid with 50-75 µL of mineral oil to prevent evaporation during subsequent amplification.

Amplifications of the control region were performed using both a water cooled PHC-2 Techne Thermocycler and a Perkin Elmer Cetus 480 DNA Thermocycler under the following conditions: initial denaturation at 94 ^{O}C for 4 minutes, annealing at 54 ^{O}C for 30 seconds, primer extension (or synthesis) for 2 minutes at 72 ^{O}C. Initial amplification was followed by forty cycles of 94 ^{O}C for 15 seconds, 54 ^{O}C for 30 seconds, and 72 ^{O}C for 2 minutes; and a final cycle of 94 ^{O}C for 15 seconds, 54 ^{O}C for 30 seconds, and 72 ^{O}C for 10 minutes. Amplifications of the ATPase-COIII products were performed using the same thermocyclers and the conditions used were identical to the control region amplification except that the annealing temperature was raised from 54 ^{o}C to 56 ^{o}C. Electrophoresis of amplified DNA was performed on a 1% agarose gel and visualized following staining with ethidium bromide under UV light.

Following amplification, PCR products were assayed for variation by restriction digestion with a battery of eight restriction enzymes for each of the amplified loci. The 1072 bp control region and the 1433 bp ATPase-COIII region were both individually digested with eight restriction enzymes (Table 3-1). Digests were performed in 20 µL volumes with 100-200 ng of amplified DNA, 3 U of enzyme and a 1/10 dilution of the manufacturer recommended 10x digestion buffer. Digestions were incubated overnight at 37^{o}C and the resulting fragments were separated by gel electrophoresis on a BioRad Vertical gel apparatus using a 4% polyacrylamide 1x TBE gel buffered in 0.5x TBE at approximately 300 volts/25 mA for 2-2.5 hours. Following electrophoresis, the restriction patterns were visualized by staining with ethidium bromide under UV light. Restriction patterns were photographed and the fragments were scored for polymorphisms. The size of each band was determined by comparison with a commercially obtained 123 bp ladder (BRL) .

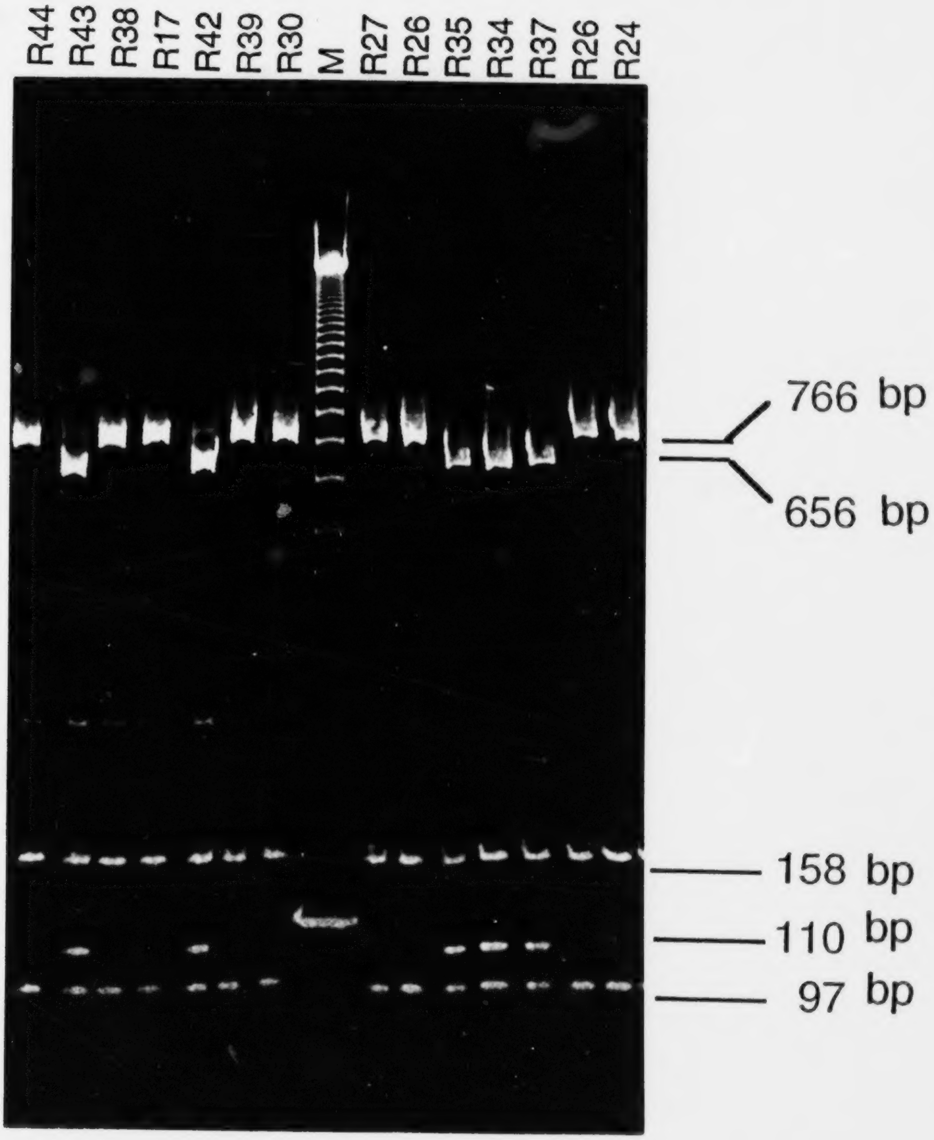
Results

Restriction digests revealed a single polymorphic site in each of the two regions (approximately 2400 bp) of salmonid mtDNA examined. A single polymorphic Sau 3A I site occurred in the control region revealing two restriction patterns; a 766 bp fragment was cut into a 656 bp fragment and a 110 bp fragment (Figure 3-2). A single polymorphic Hae III site occurred in the ATPase-6/COIII region. This revealed two restriction patterns, in which a 784 bp band was cut into two smaller bands, one at 613 bp and one at 171 bp (Figure 3-3). In both the Sau 3A I and Hae III digests several bands under 60 bp were not clearly resolved by vertical gel separation, but were verified by comparisons with the published rainbow trout sequences of Digby *et al.* (1992) and Shedlock *et al.* (1992). Restriction digests with the remaining enzymes, in both regions of rainbow trout mtDNA,

Table 3-1. The restriction enzymes used in this study. The diagonal line represents the site of cleavage in the recognition sequence, and sequences with the N designation represent any nucleotide (i.e.. A,C,G, or T).

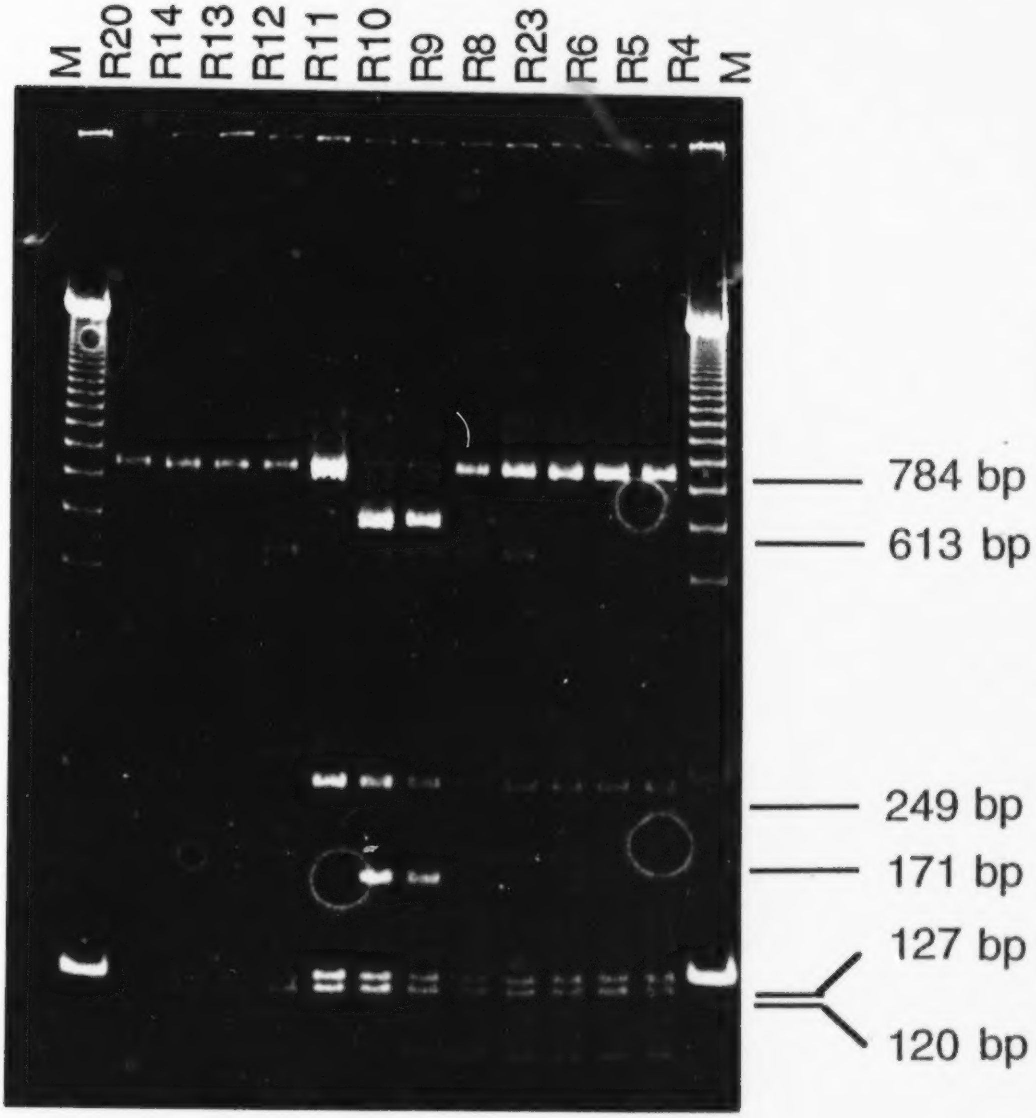
Enzyme:	Alul	Cfol	Ddel	Hae3
Cleavage site:	AG/CT	GCG/C	C/TNAG	GG/CC
Enzyme:	Hinfl	Hpa2	Rsal	Sau3a
Cleavage site:	G/ANTC	C/CGG	GT/AC	/GATC.

Figure 3-2. Restriction digestion pattern of the control region of rainbow trout mtDNA obtained using the restriction enzyme Sau 3A I. The individual identification #'s of the rainbow trout examined are indicated above the gel. All samples were sized by comparison to a commercially obtained 123 bp marker (M) and the respective bands sizes are indicated to the left of the gel image.


R44
R43
R38
R17
R42
R39
R30
M
R27
R26
R35
R34
R37
R26
R24
766 bp
656 bp
158 bp
110 bp
97 bp

Sau 3 AI

Figure 3-3. Restriction digestion pattern of the COIII-ATPase 6 region of rainbow trout mtDNA obtained using the restriction enzyme Hae III. The individual identification #'s of the rainbow trout examined are indicated above the gel. All samples were sized by comparison to a commercially obtained 123 bp marker (M) and the respective bands sizes are indicated to the left of the gel image.


M
R20
R14
R13
R12
R11
R10
R9
R8
R23
R6
R5
R4
M
784 bp
613 bp
249 bp
171 bp
127 bp
120 bp

Hae III

failed to reveal any additional polymorphic sites. Based on the presence or absence of these four restriction patterns a total of three composite rainbow trout haplotypes were identified (Table 3-2).

Despite the low numbers of individuals surveyed, the presence of only two polymorphic sites in approximately 2400 bp of the mtDNA was significantly lower than that observed in other salmonid species in Chapters 1 and 2. Fish from the Wampus Creek population of rainbow trout and the Pennask Lake Brood Stock were identical in their restriction patterns. A single individual from the Fort McMurray stock, the single fish from Pekisco Creek, the single fish from the Hinton area, and four fish from the Alison Brood Stock were identical to the Wampus Creek rainbow trout. No morphological information was available regarding the single rainbow trout from the Hinton area as only tissue was supplied for this analysis. Due to the proximity of the origin of this fish to the known Athabasca Rainbow stock in Wampus Creek, this fish may have represented a member of the local Athabasca stock. The single fish from Ohio, three fish from Fort McMurray, the two fish from the Raven Hatchery, and six fish from the Alison Creek stock were distinguishable from the Athabasca stock of rainbow trout. The most diverse group of fishwas the Alison Brood Station stock; all three haplotypes identified occurred in the individuals from the Alberta hatchery.

Table 3-2. Presence-absence of two polymorphic restriction sites in the non-coding control region and the protein coding COIII-ATPase 6 genes in the mtDNA of all rainbow trout examined in this study.

ID#	Origin	Sau 3A I	Hae III	Haplotype
R1	Fort McMurray	1	1	OmA
R2	"	1	1	OmA
R3	"	0	1	OmB
R4	"	1	1	OmA
R5	Raven Hatchery	1	1	OmA
R6	Hinton	0	1	OmB
R8	Pekisco Creek	0	1	OmB
R9	Ohio	1	0	OmC
R10	Alison Brood Station	1	0	OmC
R11	Raven Hatchery	1	1	OmA
R12	Wampus Creek	0	1	OmB
R13	"	0	1	OmB
R15	"	0	1	OmB
R16	"	0	1	OmB
R17	"	0	1	OmB
R18	"	0	1	OmB
R19	"	0	1	OmB
R20	"	0	1	OmB
R21	"	0	1	OmB
R22	"	0	1	OmB
R23	"	0	1	OmB
R24	Pennask Lake	0	1	OmB
R25	"	0	1	OmB
R26	"	0	1	OmB
R27	"	0	1	OmB
R28	"	0	1	OmB
R29	"	0	1	OmB
R30	"	0	1	OmB
R31	"	0	1	OmB
R32	"	0	1	OmB
R33	"	0	1	OmB
R34	Alison Brood Station	1	0	OmC
R35	"	1	1	OmA
R36	"	1	1	OmA
R37	"	1	0	OmC
R38	"	0	1	OmB
R39	"	0	1	OmB
R42	"	0	1	OmB
R43	"	1	1	OmA
R44	"	0	1	OmB

Discussion

Examination of amplified regions of the mtDNA using restriction enzymes failed to provide adequate resolution of the genetic differences that exist between populations of rainbow trout. Carl *et al.* (in press) examined the morphological and allozyme electrophoresis characteristics of the Athabasca Rainbow Trout and concluded that these fish are genetically distinct from all other stocks of both inland and coastal rainbow trout. My conclusions disagree with the finding of Carl *et al.* (in press), showing little difference between the mtDNA of members of the Athabasca Rainbow Trout and other rainbow trout, including two hatchery propagated strains of fish. These conclusions are in agreement with the previous findings of Wilson *et al.* (1985) and Beckenbach *et. al.* (1989) in which restriction analysis of the total mtDNA and sequence analysis of the mtDNA of a single individual Athabasca Rainbow Trout failed to distinguish it from a single individual from the Pennask stock of rainbow trout, and a single individual from the Koquihala stock of steelhead trout.

The small sample sizes and the limited genetic variation between the rainbow trout examined in this study limits the conclusions of this chapter. The shared haplotypes identified in the Wampus Creek rainbows, a possible remnant population of the Athabasca Rainbow Trout, and two of the hatchery stocks examined suggest a possible "hatchery" origin of the Wampus Creek rainbow trout. The disparity between the findings of this Chapter and the previous mtDNA studies of Wilson *et al.* (1985), and Beckenbach *et al.* (1989), and the morphological and allozyme electrophoresis analysis of Carl *et al.* (in press) suggest two scenarios. The first is that the Athabasca stock of rainbow trout in the Wampus Creek population are no different from any other stock and the differences in allozyme frequencies are incidental. Differences observed in morphology are related to the indeterminate growth rates of fishes and are a result of environmental conditions in the habitat of this population of rainbow trout. The Wampus Creek/Athabasca population of rainbow trout does not represent a unique evolutionary line but rather a unique rainbow trout morph.

The second possibility is that the lack of genetic divergence in the mtDNA of the Wampus Creek rainbow trout is a product of extensive hybridization and subsequent secondary contact with introduced rainbow trout, resulting in the swamping of the mtDNA genome with that of introduced hatchery strains of rainbow trout. In this scenario allozyme and morphological differences represent unique genetic characteristics in the nuclear DNA of individuals from the Athabasca population of rainbow trout that have persisted due to selection. Further examination of nuclear DNA would provide the necessary information to resolve this issue.

Summary

The numbers of fish examined and the limited amount of genetic divergence identified in this study severely limits the conclusions regarding the evolutionary origins and current status of the Athabasca rainbow trout. Although limited in scope this study has provided a preliminary RFLP analysis of the amount of genetic variation in the population of Athabasca Rainbow Trout. The low levels of genetic differentiation observed in the mtDNA's of the rainbow trout examined in this study indicate that any future examination of the genetic variation in rainbow trout populations would require direct sequence analysis of large numbers of individuals in order to adequately address stock and population level problems.

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General Discussion

The research presented in this thesis has examined the mtDNA diversity of several species of the family Salmonidae at the molecular, individual, population and species levels. The studies serve as one starting point for the characterization of mtDNA markers for answering stock and population level questions, and as a preliminary assessment of the levels of genetic variation that presently exists between populations of lake trout and rainbow trout in western Canada. This final section of this thesis will briefly summarize the major findings of the three chapters and suggest possible future research opportunities in the examination of the genetic diversity of fish species in western Canada.

Identification of Genetic Variation

The genetic analysis of populations of species such as lake trout and rainbow trout requires the isolation and characterization of genetic differences in homologous regions of the DNA of individual fish. In Chapter 1, the levels of genetic differentiation in two regions of the mitochondrial DNA of three species of *Salvelinus* were examined. Analysis of the sequence and structure of the noncoding control region and a region spanning the ATPase-6/COIII genes within the mtDNA of *S. namaycush*, *S. fontinalis*, and *S. confluentus*, surprisingly revealed similar amounts of sequence divergence between the noncoding and coding regions of the mtDNA in these species. In mammalian mtDNA, sequence divergence in the control region occurs at a rate 5 to 10 times that in protein coding regions (Brown *et al.*, 1979). In the case of fish mtDNA the accumulation of mutation in the noncoding region appears to be constrained at levels similar to that of the rest of the mtDNA genome. Given the equal levels of sequence divergence observed between the noncoding and the protein coding regions, and the possible ambiguities over positional homology in the alignment of non-coding regions, I concluded that protein coding regions should be favored over non-coding regions for phylogenetic and population studies of fish species.

In addition, Chapter 1 revealed some surprising phylogenetic information regarding the evolutionary relationships between the three species examined. A closer phylogenetic relationship between *S. namaycush* and *S. confluentus* than either to *S. fontinalis* was observed, contrary to the traditional pairing of *S. namaycush* and *S. fontinalis*. The process of phylogenetic inference has traditionally relied upon the analysis of morphological characteristics for the reconstruction of evolutionary relationships between groups of organisms. Molecular and DNA sequence analyses have supplied powerful tools to supplement traditional morphological examinations of taxonomic relationships.

The findings of Chapter 1 and recent examinations of the evolutionary relationships between members of the Salmonidae indicate that the phylogenetic relationships of salmonids are far from completely resolved (Blackhall, 1994 MSc. thesis; Stearly and Smith, 1993; Behnke, 1992). Future examinations of the evolutionary history of salmonid species may be undertaken at several levels; further sequence analysis of both mitochondrial and nuclear DNA of salmonid species is required to adequately address the existing problems within the systematics of the Salmonidae. For example, within the complex of rainbow trout, cutthroat trout, and the southwestern trouts are several subspecific relationships that require investigation and clarification (see Chapter 3 for references).

The occurrence of interbreeding and hybridization within the Salmonidae is another area that requires investigation. Rainbow and cutthroat trout hybridize readily when either species is introduced into the range of the other (Blackhall, MSc. thesis). The effects of the introgression of reproducing hybrid populations on the genetic integrity of these species across their ranges must be examined. Other examples of the occurrence of hybridization within the Salmonidae are the hybridization of cisco and lake whitefish in many Alberta lakes (Nelson and Paetz, 1992), the hybridization of bull trout and arctic char in their northern distribution (Wilson and Hebert, 1993), and the possible hybridization of bull and brook trout within Alberta (Roberts, 1982).

Mitochondrial DNA Diversity of Lake Trout and Rainbow Trout

The levels of genetic variation observed in *S. namaycush* populations in western Canada were comparable to those observed in the mtDNA variation among *S. namaycush* strains stocked in to Lake Ontario (Grewe, *et al.* 1993). In Chapter 2, the examination of mtDNA variation using RFLP analysis identified four haplotypes from all of the populations with no segregation of types from the ten populations examined; two or more evolutionary lineages (haplotypes identified by the RFLP analysis) were present in geographically separate populations. Further sequence analysis identified a total of 17 haplotypes and confirmed the co-occurrence of haplotypes in geographically separate populations. In the *S. namaycush* populations examined, the distributions of haplotypes suggest recent origins of these populations, with little genetic divergence between populations following their post glacial dispersion.

The distribution of mtDNA haplotypes in the examined lake trout populations may have been greatly influenced by the transfer and introduction of fish from different sources and may account for the co-occurrence of types in the populations examined. Analysis of lake trout from Cold Lake, Alberta, and three donor lakes used as source populations for the introduction of lake trout to Cold Lake revealed haplotypes that were unique to Cold Lake and Clearwater Lake, Manitoba. Based on the limited distribution of these fish, observed from our subsequent analysis of ten other lake trout populations, and the history of transfer between Cold Lake and Clearwater Lake I concluded that the introduction and recruitment of fish from Clearwater Lake to Cold Lake has occurred to some degree. Although this study met with limited success in identifying the transfer of rare types between lakes, the problem still exists of unambiguously sorting out the origins of commonly occurring types in different populations. One approach to solving this problem may be by genetically marking fish, either by cultivating rare haplotypes, or artificially inducing unique mutational changes in particular strains of lake trout. Given a unique molecular marker the recruitment and persistence of the lineages of introduced fish in recipient populations may be monitored over time. This approach would be of considerable advantage for determining the effectiveness of supplemental stocking efforts and quantifying the changes in mtDNA diversity that occur as a result of fisheries activities.

The preliminary survey of the Wampus Creek population of rainbow trout presented in Chapter 3 was of limited scope in both the number of populations examined, numbers of individuals examined, and the extent to which the genomes of individuals fish were examined. These limitations aside, the co-occurrence of haplotypes in the Wampus Creek population, and the hatchery propagated populations suggests extensive hybridization between introduced fish and the resident population of rainbow trout. Further genetic examinations of larger numbers of rainbow trout, from possible Athabasca rainbow trout populations and other resident populations in Alberta, by sequencing both mtDNA and nuclear DNA are required to adequately address the question of the uniqueness of the Athabasca stock of rainbow trout and the degree to which introduced fish have hybridized with native fish.

Development of Novel Methods

The low levels of genetic differentiation observed in both lake trout and rainbow trout emphasize the need to better understand the limitations of mtDNA-based stock discrimination. The low levels of mtDNA variation in lake trout agree with the observation of low levels of sequence variation in species from recently glaciated regions (Billington and Hebert, 1991). Although mtDNA has been successfully used to discriminate stocks of chinook salmon, America shad, smelt, walleye, and cisco (Thomas and Beckenbach, 1987; Nolan *et al.*, 1991; Taylor and Bentzen, 1993; Bernatchez and Dodson, 1990), the success of these studies can be attributed to the post-glacial founding of these populations of a few unique haplotypes and the subsequent fixation of different haplotypes that predate recent colonization. In the case of species that have recently colonized their range, such as lake trout, regional population discrimination based upon the accumulation of mutations is limited by the slow rates of divergence observed in their mtDNA and nuclear sequences.

Clearly there is a need for the development of novel technologies for the characterization of recently derived stocks of fish for the discrimination of stocks of fish. Genetic analysis of nuclear DNA offers additional information for population discrimination. The use of nuclear DNA for the above purpose has been constrained by the characteristically larger effective population size, lower levels of genetic divergence, the occurrence of recombination, and technical difficulty associated with traditional cloning and sequencing of large numbers of individuals. The use of large copy genes that contain tandemly repeated elements for DNA fingerprinting, as developed by Jeffries *et al.* (1985), has proved useful in the assessment of inbreeding rates, identification of single individuals, and family groups. Fingerprinting has been used for the above purposes in the aquaculture of tilapia (Harris *et al.*, 1991), and the stock identification of two populations of striped bass in the southeastern United States (Wrigin *et al.*, 1991). The utility of DNA fingerprinting for the large scale examinations of population and stock structure of fish species on local and regional scales is constrained by the high cost and technical difficulties associated with southern blotting. A recent modification of the original DNA fingerprinting technology of Jeffries *et al.* (1985) avoids the use of southern blotting methodology and greatly simplifies DNA fingerprinting. The most recent methodology incorporates the use of the polymerase chain reaction and the amplification of tandemly repeated elements of the nuclear genome, "microsatellites," and simple gel electrophoresis (see Bryford and Wayne, 1993 for a comprehensive review).

The value of microsatellite analysis for population discrimination is unparalleled, but the utility of this technology for phylogenetic analysis of divergent evolutionary groups is limited. High levels of genetic divergence in microsatellite DNA within a species may lead to ambiguous conclusions regarding the evolutionary boundaries between species. For example microsatellite analyses of individual black bears from the geographic extremes of their range were as genetically distinct from each other as they were to polar bears (D. Paetkow personal communication). The inability of microsatellite analysis to provide phylogenetic information that mtDNA analysis can readily provide ensures the continued use of both of these techniques for the genetic analysis of fish stocks. Microsatellite analysis of large numbers of individuals has been accomplished for several mammalian and insect species, but few fish species (Rico *et al.*, 1993; Bruford and Wayne, 1993). Development of this technology for stock identification of fish species such as lake trout and rainbow trout may provide answers where mtDNA analysis can only raise questions; the combination of the two technologies provides a powerful strategy for the future preservation and management of fish species.

The tools to examine the genetic diversity of large numbers of fish species are now available. It is recognized that the identification and characterization of genetically distinct populations are vital to understanding the genetic diversity of fishes. Past interests in the genetic diversity of fish species had been concerned primarily with the hatchery propagation of fish (Allendorf, 1987). The effect of fisheries manipulation upon the genetics of wild fish stocks was largely ignored. Due to the reckless history of manipulating fish stocks, current management decisions should be based upon facts rather than speculations about the origins and relationships between populations, stocks, and species. An understanding of the genetic structure of fish populations and the impacts of fish management practices such as introductions and harvesting upon the genetic structure of these populations is vital for the optimization of fish management and preservation. Mayhood (1992) recommended the genetic characterization and taxonomic analysis of 14 of 19 fish species listed in a preliminary assessment of the fish stocks of Jasper National Park. Similar requirements exist for the development of any effective fisheries management strategy. The paucity of genetic information on the population structure, stock structure, and taxonomic relationships of freshwater and marine species of fish in Canada and the surrounding regions requires further genetic examination to adequately understand the underlying biological principles of this resource.

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Digest Summary

The following table summarizes the presence-absence of all restriction patterns observed for all lake char individuals examined from the nine study populations. Four restriction enzymes were found to be polymorphic and are denoted by an *. The comprehensive haplotype for each individual are listed in the last column and the sample identification number is listed in the second column.

		*	*	*	*					
Sample origin:	ID#	HinfI	HaeIII	Sau3a	AluI	RsaI	CfoI	HpaII	DdeI	Htype
Cornwall L., Alberta	Sn1	1	0	1	0	0	0	0	0	A
N = 8	Sn2	1	0	1	0	0	0	0	0	A
	Sn3	1	0	1	0	0	0	0	0	A
	Sn4	1	0	1	0	0	0	0	0	A
	Sn5	1	0	1	0	0	0	0	0	A
	Sn6	1	0	1	0	0	0	0	0	A
	Sn7	1	0	1	0	0	0	0	0	A
	Sn8	1	0	1	0	0	0	0	0	A
Clearwater L. Manitoba	Sn10	1	0	1	0	0	0	0	0	A
N = 23	Sn11	1	0	0	0	0	0	0	0	C
	Sn12	1	0	1	0	0	0	0	0	A
	Sn14	1	0	0	0	0	0	0	0	C
	Sn15	0	1	0	0	0	0	0	0	B
	Sn16	0	1	0	0	0	0	0	0	B
	Sn17	1	0	1	0	0	0	0	0	A
	Sn18	0	1	0	0	0	0	0	0	B
	Sn74	1	0	0	0	0	0	0	0	C
	Sn75	1	0	1	0	0	0	0	0	A
	Sn76	1	0	1	0	0	0	0	0	A
	Sn77	0	1	0	0	0	0	0	0	B
	Sn78	1	0	0	0	0	0	0	0	C
	Sn79	1	0	1	0	0	0	0	0	A
	Sn80	1	0	1	0	0	0	0	0	A
	Sn81	1	0	1	0	0	0	0	0	A
	Sn82	1	0	0	0	0	0	0	0	C
	Sn84	0	1	0	0	0	0	0	0	B
	Sn86	0	1	0	0	0	0	0	0	B
	Sn87	1	0	0	0	0	0	0	0	C
	Sn91	1	0	0	0	0	0	0	0	C
	Sn92	0	1	0	1	0	0	0	0	D
	Sn93	1	0	1	0	0	0	0	0	A
Clearwater L., BNP	Sn133	0	1	0	0	0	0	0	0	B
n=4	Sn134	0	1	0	0	0	0	0	0	B
	Sn135	0	1	0	0	0	0	0	0	B
	Sn136	0	1	0	0	0	0	0	0	B

CoIII Atpase 8 Digest summary, CONT. (*polymorphic loci):

		*	*	*	*					
Sample origin:	ID#	HinfI	HaeIII	Sau3a	AluI	RsaI	CfoI	HpaII	DdeI	Htype
White Swan L., Sask.	Sn19	0	1	0	0	0	0	0	0	B
	Sn21	1	0	1	0	0	0	0	0	A
n=26	Sn23	1	0	0	0	0	0	0	0	B
	Sn24	1	0	0	0	0	0	0	0	C
	Sn25	1	0	1	0	0	0	0	0	A
	Sn26	1	0	0	0	0	0	0	0	C
	Sn27	1	0	1	0	0	0	0	0	A
	Sn94	1	0	1	0	0	0	0	0	A
	Sn95	1	0	1	0	0	0	0	0	A
	Sn96	1	0	1	0	0	0	0	0	A
	Sn97	0	1	0	0	0	0	0	0	B
	Sn99	0	1	0	0	0	0	0	0	B
	Sn100	1	0	1	0	0	0	0	0	A
	Sn101	0	1	0	0	0	0	0	0	B
	Sn102	0	1	0	0	0	0	0	0	B
	Sn103	0	1	0	0	0	0	0	0	B
	Sn104	0	1	0	0	0	0	0	0	B
	Sn105	1	0	1	0	0	0	0	0	A
	Sn107	1	0	1	0	0	0	0	0	A
	Sn108	1	0	1	0	0	0	0	0	A
	Sn109	0	1	0	0	0	0	0	0	B
	Sn110	0	1	0	0	0	0	0	0	B
	Sn111	1	0	1	0	0	0	0	0	A
	Sn112	1	0	1	0	0	0	0	0	A
	Sn113	0	1	0	0	0	0	0	0	B
	Sn114	0	1	0	0	0	0	0	0	B
Cold L., Alberta	Sn37	1	0	1	0	0	0	0	0	A
Adults	Sn38	0	1	0	0	0	0	0	0	B
N = 17	Sn39	0	1	0	1	0	0	0	0	D
	Sn40	1	0	1	0	0	0	0	0	A
	Sn41	0	1	0	0	0	0	0	0	B
	Sn42	1	0	1	0	0	0	0	0	A
	Sn43	0	1	0	0	0	0	0	0	B
	Sn44	0	1	0	0	0	0	0	0	B
	Sn45	0	1	0	0	0	0	0	0	B
	Sn58	1	0	1	0	0	0	0	0	A
	Sn59	0	1	0	0	0	0	0	0	B
	Sn61	1	0	1	0	0	0	0	0	A
	Sn62	0	1	0	0	0	0	0	0	B
	Sn63	0	1	0	0	0	0	0	0	B
	Sn64	1	0	1	0	0	0	0	0	A
	Sn65	0	1	0	0	0	0	0	0	B
	Sn66	1	0	1	0	0	0	0	0	A
Cold L., Alberta	Sn28	1	0	1	0	0	0	0	0	A
Subadults	Sn29	1	0	1	0	0	0	0	0	A
N = 14	Sn30	0	1	0	0	0	0	0	0	B
	Sn31	1	0	1	0	0	0	0	0	A
	Sn32	1	0	1	0	0	0	0	0	A
	Sn34	1	0	1	0	0	0	0	0	A
	Sn35	1	0	1	0	0	0	0	0	A

CoIII Atpase 8 Digest summary, CONT. (*polymorphic loci):

Sample origin:	ID#	* HinfI	* HaeIII	* Sau3a	* AluI	RsaI	CfoI	HpaII	DdeI	Htype
Cold L., Alberta	Sn36	0	1	0	1	0	0	0	0	D
Subadults	Sn67	0	1	0	1	0	0	0	0	D
N = 14	Sn68	0	1	0	0	0	0	0	0	B
	Sn69	1	0	1	0	0	0	0	0	A
	Sn70	1	0	0	0	0	0	0	0	C
	Sn71	1	0	1	0	0	0	0	0	A
	Sn72	0	1	0	1	0	0	0	0	D
Cold L., Alberta	Sn46	0	1	0	1	0	0	0	0	D
Juviniles	Sn47	1	0	1	0	0	0	0	0	A
N = 12	Sn48	1	0	1	0	0	0	0	0	A
	Sn49	1	0	1	0	0	0	0	0	A
	Sn50	0	1	0	0	0	0	0	0	B
	Sn51	1	0	1	0	0	0	0	0	A
	Sn52	0	1	0	0	0	0	0	0	B
	Sn53	0	1	0	1	0	0	0	0	D
	Sn54	1	0	1	0	0	0	0	0	A
	Sn55	0	1	0	0	0	0	0	0	B
	Sn56	1	0	1	0	0	0	0	0	A
	Sn57	1	0	1	0	0	0	0	0	A
Pyramid L., JNP	P1	1	0	1	0	0	0	0	0	A
n=9	P2	1	0	1	0	0	0	0	0	A
	P3	0	1	0	0	0	0	0	0	B
	P4	1	0	0	0	0	0	0	0	C
	P5	1	0	0	0	0	0	0	0	C
	P6	1	0	0	0	0	0	0	0	C
	P7	1	0	1	0	0	0	0	0	A
	P8	0	1	0	0	0	0	0	0	B
	P9	1	0	0	0	0	0	0	0	C
Sassenach L., JNP	Sn115	1	0	1	0	0	0	0	0	A
n=15	Sn116	1	0	1	0	0	0	0	0	A
	Sn117	1	0	1	0	0	0	0	0	A
	Sn118	1	0	1	0	0	0	0	0	A
	Sn119	1	0	1	0	0	0	0	0	A
	Sn120	1	0	1	0	0	0	0	0	A
	Sn121	1	0	1	0	0	0	0	0	A
	Sn122	1	0	1	0	0	0	0	0	A
	Sn123	1	0	1	0	0	0	0	0	A
	Sn124	1	0	1	0	0	0	0	0	A
	Sn125	1	0	1	0	0	0	0	0	A
	Sn145	1	0	1	0	0	0	0	0	A
	Sn146	1	0	1	0	0	0	0	0	A
	Sn182	1	0	1	0	0	0	0	0	A
	Sn183	1	0	1	0	0	0	0	0	A
Glacier L., BNP	Sn126	1	0	1	0	0	0	0	0	A
n=15	Sn127	1	0	1	0	0	0	0	0	A
	Sn128	1	0	1	0	0	0	0	0	A
	Sn129	1	0	1	0	0	0	0	0	A
	Sn130	1	0	1	0	0	0	0	0	A

CoIII Atpase 8 Digest summary, CONT. (*polymorphic loci):

		*	*	*	*					
Sample origin:	ID#	HinfI	HaeIII	Sau3a	AluI	RsaI	CfoI	HpaII	DdeI	Htype
Glacier L., BNP	Sn131	1	0	1	0	0	0	0	0	A
n=15	Sn132	1	0	1	0	0	0	0	0	A
	Sn137	1	0	1	0	0	0	0	0	A
	Sn138	1	0	1	0	0	0	0	0	A
	Sn139	1	0	1	0	0	0	0	0	A
	Sn140	1	0	1	0	0	0	0	0	A
	Sn141	1	0	1	0	0	0	0	0	A
	Sn142	1	0	1	0	0	0	0	0	A
	Sn143	1	0	1	0	0	0	0	0	A
	Sn144	1	0	1	0	0	0	0	0	A
Peerles L., Alberta	Sn150	0	1	0	0	0	0	0	0	B
n=11	Sn151	0	1	0	0	0	0	0	0	B
	Sn153	0	1	0	0	0	0	0	0	B
	Sn154	0	1	0	0	0	0	0	0	B
	Sn155	1	0	1	0	0	0	0	0	A
	Sn156	0	1	0	0	0	0	0	0	B
	Sn157	1	0	1	0	0	0	0	0	A
	Sn158	0	1	0	0	0	0	0	0	B
	Sn159	0	1	0	0	0	0	0	0	B
	Sn160	0	1	0	0	0	0	0	0	B
	Sn161	1	0	0	0	0	0	0	0	A
Minnewanka, BNP	Sn162	1	0	1	0	0	0	0	0	A
n=19	Sn163	1	0	0	0	0	0	0	0	C
	Sn164	1	0	1	0	0	0	0	0	A
	Sn165	1	0	1	0	0	0	0	0	A
	Sn166	1	0	1	0	0	0	0	0	A
	Sn167	1	0	1	0	0	0	0	0	A
	Sn168	1	0	1	0	0	0	0	0	A
	Sn169	1	0	1	0	0	0	0	0	A
	Sn170	1	0	1	0	0	0	0	0	A
	Sn171	1	0	1	0	0	0	0	0	A
	Sn172	1	0	1	0	0	0	0	0	A
	Sn173	1	0	0	0	0	0	0	0	C
	Sn174	1	0	1	0	0	0	0	0	A
	Sn175	1	0	1	0	0	0	0	0	A
	Sn177	1	0	1	0	0	0	0	0	A
	Sn178	1	0	0	0	0	0	0	0	C
	Sn179	1	0	0	0	0	0	0	0	C
	Sn180	1	0	0	0	0	0	0	0	C
	Sn181	1	0	0	0	0	0	0	0	C